1993

Regulation Of Gene Expression Of Surfactant-associated Proteins In Developing Rabbit Lung

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REGULATION OF GENE EXPRESSION OF SURFACTANT-ASSOCIATED PROTEINS IN DEVELOPING RABBIT LUNG

by

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Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
the University of Western Ontario
London, Ontario
March 1993

ISBN 0-315-83979-1

ABSTRACT

Pulmonary surfactant replacement therapy and antenatal glucocorticoid administration are the two treatment strategies used for Respiratory Distress Syndrome. Because of the important roles surfactant-associated proteins play in surfactant function, understanding the mechanisms involved in regulation of the genes encoding for the proteins in fetal development or upon glucocorticoid treatment is therefore of paramount importance in the overall design of an effective treatment regimen. We attempted in this project to investigate the developmental and glucocorticoid regulation of three surfactant proteins, SP-A, SP-B and SP-C, using the rabbit as a model. The mRNA levels for these three proteins were examined in developing rabbit lung. By measuring both mRNA accumulation and synthesis, using Northern and slot blot analysis and nuclear transcription run-on assays, respectively, we were able to show that the genes encoding the three proteins were regulated independently with respect to the initiation of gene expression and changing patterns in either transcriptional or mRNA accumulation during perinatal period in rabbit lung. Effects of in vitro glucocorticoid treatment on mRNA levels and the relative transcriptional activities of the SP-A, SP-B and SP-C genes were also examined in an explant system using lung tissues from two different gestational age fetuses. It was observed that (1) glucocorticoids accelerated SP-B mRNA levels but not gene transcription, implying an apparent involvement of post-transcriptional mechanisms; (2) the hormone had a dose-dependent biphasic effect on both SP-A mRNA accumulation and synthesis, however, dexamethasone at 10^{-8}M showed no apparent influence on SP-A

mRNA production as a function of incubation time, indicating also a dose-dependent (perhaps age-dependent too) responsiveness of the SP-A gene to glucocorticoid; and (3) there was a dose-dependent response to in vitro dexamethasone treatment of SP-C gene transcription but not mRNA levels. When examined as a function of incubation time, 10^{-8}M dexamethasone showed a more profound effect on the SP-C mRNA levels with 26-day lungs. These suggested the involvement of both transcriptional and post-transcriptional regulation of the SP-C gene by in vitro glucocorticoid treatment.

To my parents

ACKNOWLEDGMENT

I would like to express my sincere appreciation to my supervisor, Dr. Fred Possmayer, for his guidance, encouragement and support during the course of this work and for his proof reading on my thesis. I would also like to thank my advisory committee, Drs. Geoff Hammond and George Chaconas, for their guidance and constructive criticisms. Drs. George Mackie and Bill McMurray deserve acknowledgment for offering excellent suggestions, inspiration and encouragement.

Special thanks go to Drs. Li-juan Yao and Jesse Shen for their friendship, family-like understanding and support throughout my endeavours. Ms Carol Richardson and Ms Carol Ford deserve sincere appreciation for introducing me into molecular biology. Thank you to all the people of or associated with Dr. Possmayer's lab in the past, Dr. Mathi Nagappan, Mr. Mark Quirie, Mr. Ian Connelly and Dr. Amanda Cockshutt and in the present, Drs. Shou-Hwa Yu, Octavio Filguerias, Ruud Veldhuizen, Dave Bjarneson, Cheryl Ketola, Mr. Kevin Inchley, Mr. Ovidio Coto, Mr. Gerry Barbe and Mrs. Mary Ann Omseth, Ms Barb Lowery, Ms. Joan Barber and Mrs. Elizabeth Marak. A special thank goes to Riad Qanbar for her friendship and most kindly help in typing my thesis. All your friendship will always be treasured.

The financial support of The University of Western Ontario is gratefully acknowledged. As well, Dr. Possmayer provided financial support through his grant from the Natural Sciences and Engineering Research Council.

Lastly, I would like to express my deepest gratitude to my family, my parents and

my little brother, for their never-ending love and constant inspiration and encouragement. None of this would have been possible without the extraordinary support I have received from them. The understanding and encouragement from my former supervisor, Dr. Renhua Zhang of Jinan University, will always be remembered.

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LIST OF ABBREVIATIONS

ARDS	adult respiratory distress syndrome
ActD	actinomycin D
bp	base pairs
BMV	Brome mosaic virus
BSA	bovine serum albumin
°C	degree Celsius
cAMP	adenosine 3',5'-monophosphate
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
CHX	cycloheximide
cpm	counts per minute
cRNA	complementary RNA
CsCl	cesium chloride
C-terminus	carboxyl-terminus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DEX	dexamethasone
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor

g	gravity
h	hour(s)
kb	kilobasepair(s)
kDa	kilodalton(s)
N-terminus	amino-terminus
NTP	ribonucleotide triphosphate
NRDS	neonatal respiratory distress syndrome
M	molar
μg	microgram(s)
mg	milligram(s)
min	minute(s)
μl	microlitter(s)
ml	millilitter(s)
mM	millimolar
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonylfluoride
pmole	picomole
poly(A)	polyadenylate

RNA	ribonucleic acid
RDS	respiratory distress syndrome
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TCA	trichloride acetic acid
TGF	transforming growth factor
TNF	tumour necrosis factor
TPA	12-*o*-tetradecanoylphorbol-13-acetate
Tris	tris(hydroxymethyl)aminomethane
U	units
UV	ultraviolet
UTP	uridine triphosphate

CHAPTER 1 - GENERAL INTRODUCTION

1.1. Pulmonary Surfactant

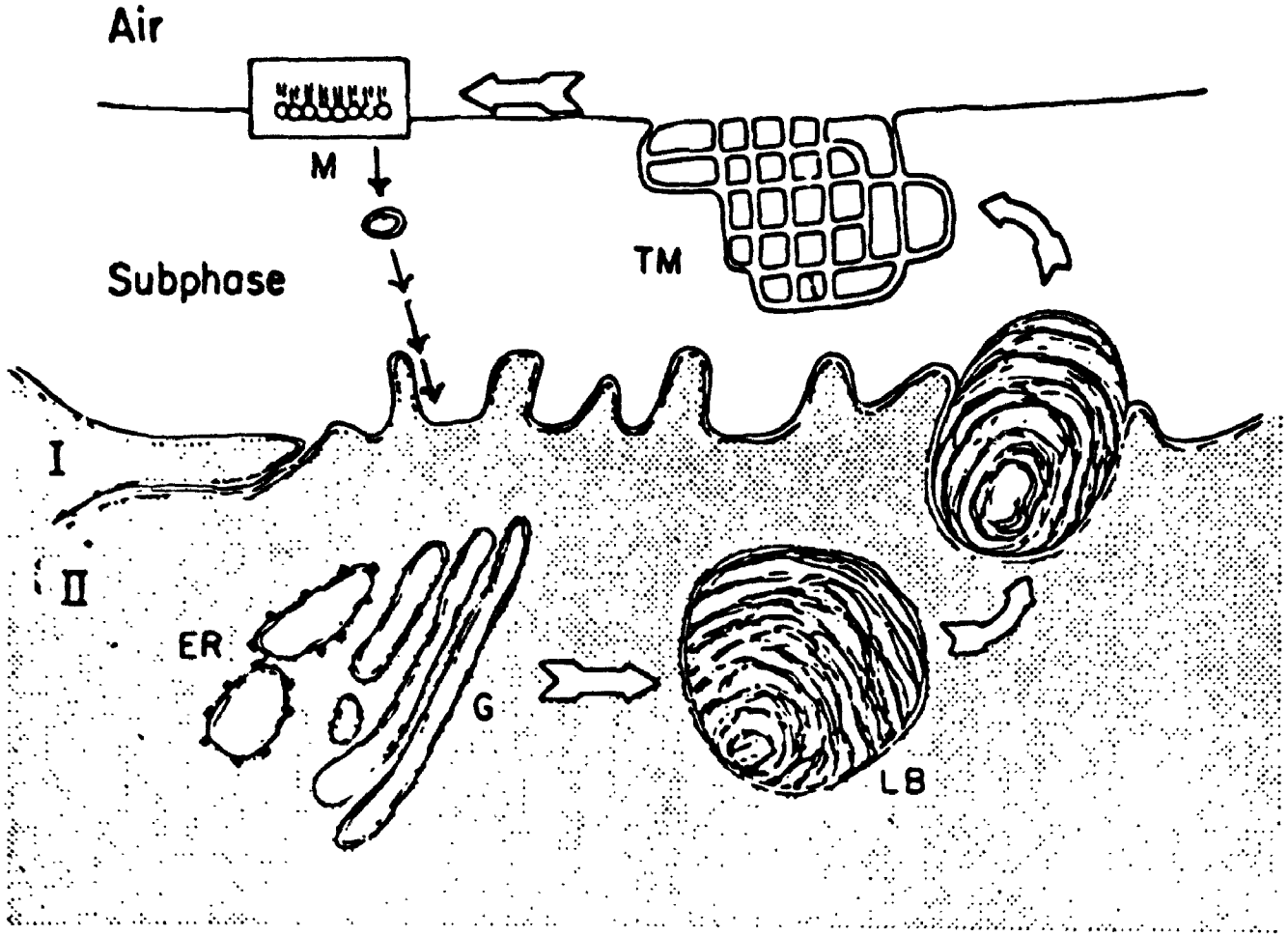
1.1.1 General Introduction

Pulmonary surfactant is a complex mixture of lipids and proteins found in the alveolar spaces and terminal airways of the lung which reduces the surface tension across the air-liquid interface. Despite the complex nature of surfactant, it is thought that a monolayer enriched in the disaturated phospholipid, dipalmitoylphosphatidylcholine (DPPC), is responsible for the reduction of surface tension to very low values upon expiration (Clements, 1977; Notter, 1984a; von Golde et al., 1984; Cockshutt & Possmayer, 1992). The other components of surfactant, including the unsaturated and anionic phospholipids and the surfactant-associated proteins, are required to generate and maintain this monolayer highly enriched in DPPC.

It is now generally accepted that surfactant components are synthesized by the alveolar type II cell. Surfactant is assembled and stored within the type II cell in lamellar bodies, the intracellular pool of surfactant, and released by exocytosis into the alveolar lumen (Fig.1.1). In the alveolar space this lamellar structure can be transformed into the lattice-like structure, tubular myelin, which may represent the immediate precursor of the surface monolayer film (Goerke, 1974). The monolayer lines the alveolus and by reducing the surface tension of the aqueous-lining layer prevents the collapse of alveoli during the expiratory phase of respiration.

Surfactant production is under developmental and hormonal control (see below).

Figure 1.1. Schematic diagram of the surfactant system. A portion of an alveolus is shown with the location and movement of surfactant components depicted. Surfactant components are synthesized by type II cell (II) in the endoplasmic reticulum (ER) and transported via the Golgi apparatus (G) to lamellar bodies (LB). After secretion into the liquid lining subphase in the alveolus, the surfactant forms tubular myelin (TM), which is thought to be the direct precursor of the phospholipid monolayer (M). This monolayer is responsible for the surface tension properties of surfactant. Subsequently, surfactant components are taken back into type II cells, perhaps in the form of small vesicles. (From Possmayer et al., 1984, modified from Goerke, 1974.)

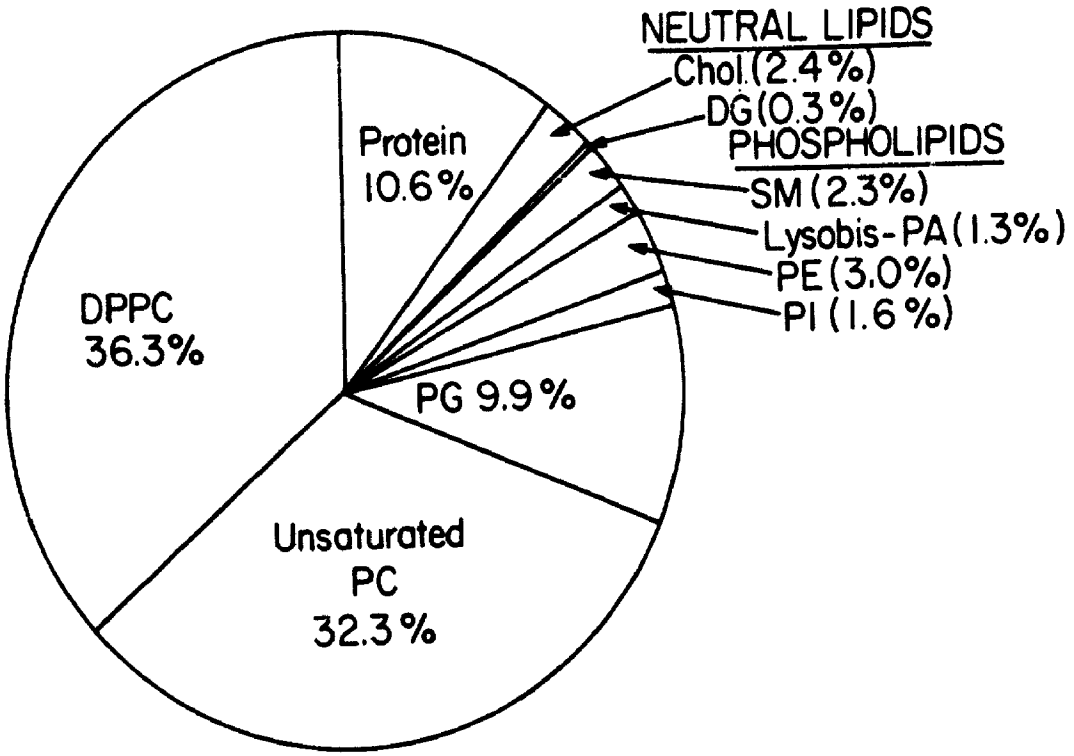

Air
M
Subphase
TM
I
II
ER
G
LB

Delivery of infants prior to completion of lung maturation can result in surfactant deficiency which leads to collapse of the alveoli (atelectasis) and the development of the Respiratory Distress Syndrome (RDS) (Avery & Mead, 1959), the leading cause of death in premature newborns in developed countries. Another pathological situation, adult respiratory distress syndrome (ARDS), may also be accompanied by alterations in surfactant function (Holm & Matalon, 1989; Jobe, 1989; Seeger et al., 1990; Lewis and Jobe, 1993). Recent clinical trials have shown that surfactant replacement is an effective therapy for diseases characterized by surfactant deficiency or inactivation (Jobe & Ikegami, 1987; Collaborative Groups, 1988; Robertson & Lachmann, 1988; Ballard et al., 1992). Further progress in the treatment of these diseases will depend on a better understanding of the processes involved in regulating the synthesis, secretion and metabolism of pulmonary surfactant.

1.1.2. Composition of pulmonary surfactant

Surfactant purified from lung lavage is composed of approximately 90% lipids and 10% proteins. The composition is highly conserved among species (Shelly et al., 1984; Yu et al., 1983; Possmayer et al., 1984). Figure 1.2 is a schematic depiction of the composition of bovine surfactant obtained by centrifugation of lavage material (Yu et al., 1983). The protein fraction contains the surfactant-associated proteins (discussed below). The lipid fraction is ~97% phospholipid and ~3% neutral lipid. The neutral lipid in bovine surfactant is mostly cholesterol (Chol) (~90%), with the remainder being mainly diacylglycerol (DG). The amount of neutral lipid varies between species and may

Figure 1.2. The composition of bovine pulmonary surfactant. The percentage by weight of the surfactant components is represented. Data obtained from Yu et al, 1983. Abbreviations: DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; Lysobis-PA, lyso-*bis*-phosphatidic acid; SM, sphingomyelin; DG, diacylglycerol; Chol, cholesterol.



COMPOSITION OF BOVINE PULMONARY SURFACTANT

depend to some extent on the purification protocol (for review see Possmayer et al., 1984). The phospholipid fraction is predominantly phosphatidylcholine (PC), representing ~80%, and phosphatidylglycerol (PG) representing ~11%, of the total phospholipid. The remainder includes small amounts of phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylinositol (PI), and lyso-*bis*-phosphatidic acid (lysobis PA).

Over half of the phosphatidylcholine is disaturated (containing saturated fatty acid at both the *sn*1- and 2- positions). The bulk of the remaining PC is monoenoic. Surfactant also contains some disaturated PG.

As stated earlier, dipalmitoylphosphatidylcholine (DPPC) is the primary surface tension reducing agent of the surfactant complex. However, by themselves, surfactant phospholipids adsorb and spread very slowly to an aqueous surface. It has been shown that the protein components of surfactant greatly enhance the rate of adsorption and spreading of surfactant phospholipids (Possmayer, 1990) and may also be involved in the squeeze-out of other surfactant components resulting in a monolayer enriched with DPPC (Yu & Possmayer, 1992). In addition, these proteins have also been shown to regulate surfactant phospholipid synthesis and secretion by purified type II cells (Thakur et al., 1986; Rice et al., 1987; Dobbs et al., 1987) (see section 1.1.4).

1.1.3. Identification of surfactant-associated proteins in bronchiolar lavage fluid

The proteins which are isolated specifically with pulmonary surfactant have been given the designation surfactant-associated protein, or SP- (Possmayer, 1988).

Pulmonary surfactant is isolated by differential centrifugation of bronchoalveolar lavage fluid. A large number of alveolar macrophages, which are also recovered by the lavage procedure, are removed by centrifugation at low speed. The supernatant, after a subsequent high-speed centrifugation, yields natural surfactant. Extraction of natural surfactant into organic solvents leaves behind the major surfactant-associated protein referred to as SP-A and another large-mass hydrophilic protein SP-D. Two extremely hydrophobic proteins, SP-B and SP-C, remain in the organic phase.

1.1.4. Structures, properties and functions of surfactant-associated proteins

1.1.4.1. SP-A.

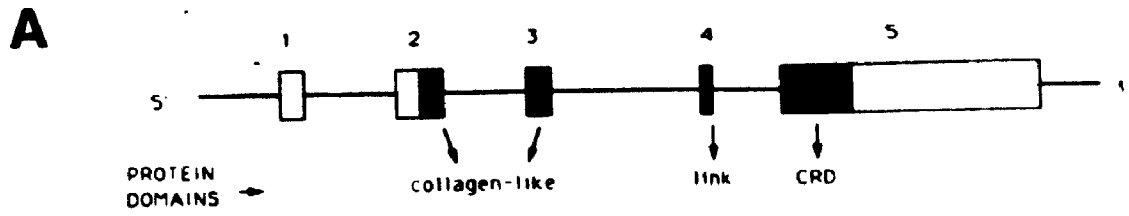
SP-A, the most abundant surfactant-associated protein, was the first to be discovered and is the best characterized. This protein is a large, complex glycoprotein (King et al., 1989), with a monomeric molecular weight of 26-36 kDa depending on the species and extent of glycosylation (Weaver and Whitsett, 1991).

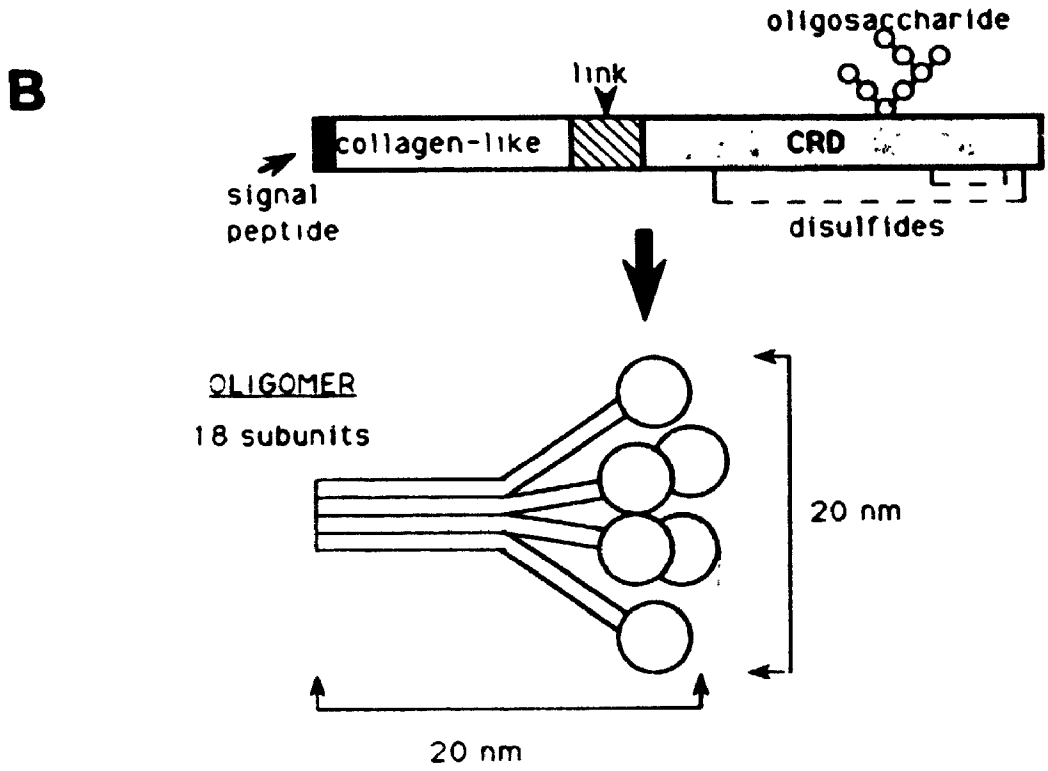
Human SP-A is a protein of 248 amino acids (White et al., 1985). It arises from an mRNA species of ~2.2 kb (Hawgood, 1992). Both rat and rabbit lung contain 2 species of SP-A RNA which arise from alternative polyadenylation sites (Boggaram et al., 1988a, 1988b; Sano et al., 1987). The primary translation product of SP-A undergoes a variety of post-translational modifications including formation of intra- and intermolecular disulphide bonds, hydroxylation of the proline residues in the collagen-like region and possibly carboxylation at an asparagine residue (for review see Weaver, 1992b).

The predicted amino acid sequence of SP-A as well as rotary shadowing electron microscopy of the purified protein have shown that the SP-A monomer consists of two structurial domains, a globular head (C-terminal) and a tail region (N-terminal) (Fig.1.3) (Hawgood et al., 1989; Voss et al., 1988). The head region is glycosylated and has sequence homology with several lectins. The tail region has homology to collagen in that it has 23 or 24 repeats of the Gly-X-Y triplet, where X and Y are often proline or hydroxyproline. This region extends for nearly one-third of the length of the molecule from near the N-terminus (White et al., 1985) and forms a triple helix with two other SP-A monomers (Voss et al., 1988). Between the two regions there is a stretch of 30-40 amino acids of hydrophobic linker which may possess the SP-A lipid-binding ability (Ross, 1986, 1991) (see below). Non-denatured SP-A is an oligomer of 18 subunits (Voss et al., 1988) with a molecular weight of approx. 650 kDa (King et al., 1989). It has recently been demonstrated by rotary shadowing electron microscopy that six of the SP-A trimers were associated to form a rosette-like molecule (Voss et al., 1988) (Fig. 1.3).

In bronchoalveolar lavage fluid SP-A is found to be tightly associated with surfactant phospholipids. Purified SP-A binds to many phospholipids in vitro (King et al., 1986; Kuroki et al., 1991c). A specific interaction has been observed between SP-A and DPPC (Kuroki et al., 1991c). SP-A alone, however, has little effect on the surface activity of phospholipids (Hawgood et al., 1987). In the presence of the two hydrophobic surfactant proteins SP-B and SP-C, SP-A markedly enhances the surface activity of phospholipids (Hawgood et al., 1987; King et al., 1983). Morphological

Figure 1.3. Gene and protein structure of SP-A. (A) Schematic organization of the SP-A gene. The human SP-A gene is approximately 4.5 kb long and comprised of 5 exons (boxes) and 4 introns (lines). Coding sequence is indicated by the filled boxes. The relationship of the exon organization to the major protein domain is shown. (B) Schematic organization of SP-A. The major domains of the protein are indicated on a linear representation of a monomeric subunit. The carbohydrate recognition domain (CRD) is the globular region noted in the text. The collagen-like domain is the tail region which is involved in triple helix formation as stated in the text. The drawing is taken from Hawgood (1992) with permission.


A
1
2
3
4
5
5'
PROTEIN DOMAINS
collagen-like
link
CRD


B
oligosaccharide
link
collagen-like
CRD
signal peptide
disulfides
OLIGOMER
18 subunits
20 nm
20 nm

studies also reveal that SP-A alone induces the aggregation of phospholipid but in the presence of SP-B there is a further dramatic structural rearrangement with the formation of a lattice-like structure similar to the native tubular myelin structure (Suzuki et al., 1989; Williams et al., 1991; Efrati et al., 1987; Hook, 1986).

SP-A is also a calcium-binding protein. It has been shown that SP-A contains at least two distinct binding sites for calcium (Haagsman et al., 1990). Occupancy of the different sites may induce distinct conformational changes and biological effects. SP-A is dissociated from the native pulmonary surfactant complex in the absence of calcium (Benson et al., 1984). Calcium is required for the ability of SP-A to enhance surface activity of phospholipids with other surfactant proteins and for the carbohydrate-binding capacity of this protein (see below).

It is known that SP-A binds to monosaccharides in vitro (Haagsman et al., 1987). The binding is calcium-dependent and requires intact intrachain disulfide bonds (Haagsman et al., 1987). Recently it has been suggested SP-A actually binds to oligosaccharide attached to other SP-A molecules (Haagsman et al., 1991). Calcium-dependent "self-recognition" may therefore have an important role in mediating the SP-A induced phospholipid aggregation (Efrati et al., 1987) and the structural rearrangement thought to be important in the formation of tubular myelin (Efrati et al., 1987; Hook et al., 1986; Suzuki et al., 1989; Williams et al., 1991). Antibody labelling studies suggest SP-A may be localized in the corners of the tubular myelin lattice (Voorhout et al., 1991). The in vitro reconstitution of structures similar to tubular myelin is dependent on the presence of SP-A (Suzuki et al., 1989; Williams et al., 1991). No studies,

however, have yet established that SP-A is necessary to maintain a functional lipid-rich surface film and therefore alveolar stability in the normal lung.

Binding of SP-A to oligosaccharide may also be involved in this protein's role in non-antibody-mediated defense against micro-organisms. Purified SP-A appears to interact with macrophages in culture (Manz-Keinker et al., 1991) in ways that stimulate phagocytosis of opsonized particles, bacteria (Tenner et al., 1989; van Iwaarden et al., 1990) and viruses (van Iwaarden et al., 1991).

Studies with isolated cells also suggest SP-A has a role in maintaining homeostasis between extracellular and intracellular surfactant pools. SP-A both increases the uptake of DPPC into type II cells in vitro and decreases the stimulated secretion of lamellar bodies from these cells (Dobbs et al., 1987; Rice et al., 1987; Kuroki et al., 1988a). The characteristics of these effects and the nature of the binding of SP-A to both type II cells and alveolar macrophages are consistent with the presence of specific SP-A receptors on these cells (Kuroki et al., 1988a, 1988b).

1.1.4.2. SP-B and SP-C.

Two, low molecular weight hydrophobic surfactant-associated proteins, SP-B and SP-C have been purified and partially characterized. Both proteins are extremely hydrophobic, extract with lipid into organic solvents such as chloroform/methanol (Yu et al., 1983) and coelute with PG and PE during subsequent silicic acid column chromatography (Yu & Possmayer, 1988, 1989; Possmayer, 1990).

The larger of the two peptides, SP-B, appears as a protein of M_r= 8,000

following SDS/PAGE under reducing conditions. Amino acid sequence analysis identified bovine SP-B as a peptide of 79 residues (Olafson et al., 1987; Johansson et al., 1988) which is generated by translation of a 2.0 kb mRNA and subsequent proteolytic processing of an approx. 40 kDa primary translation product (Fig.1.4) (Jacobs et al., 1987). The SP-B precursor contains a hydrophobic leader sequence for the secretion of the preproprotein by processes independent of glycosylation (O'Reilly et al., 1989a). The preproprotein has a glycosylation site near the C-terminal in all species studied. The active SP-B mature peptide contains no carbohydrate. Its sequence deduced from the cDNA is very hydrophobic, consistent with the solubility of SP-B in organic solvents. Examination of proprotein processing in a human adenocarcinoma cell line (H441) revealed that SP-B peptides of M_r=27,000-33,000 and M_r=16,000, representing carboxyl and amino-terminal domains, accumulate in the media. The N-terminal region of the 27,000 fragment contains the active SP-B peptide (O'Reilly et al., 1989a).

SP-B purified from bronchoalveolar lavage fluid occurs as thiol-dependent oligomers with the dimer being the predominant form in species studied to date. It is likely that sulfhydryl and non-sulfhydryl-dependent aggregation generate the larger SP-B oligomers present in surfactant. The size of the oligomers varies among species studied. SP-B from dog and rat migrates at M_r = 18,000, but bovine SP-B contains forms which migrate at M_r= 24,000 - 36,000 (for review see Whitsett and Baatz, 1992).

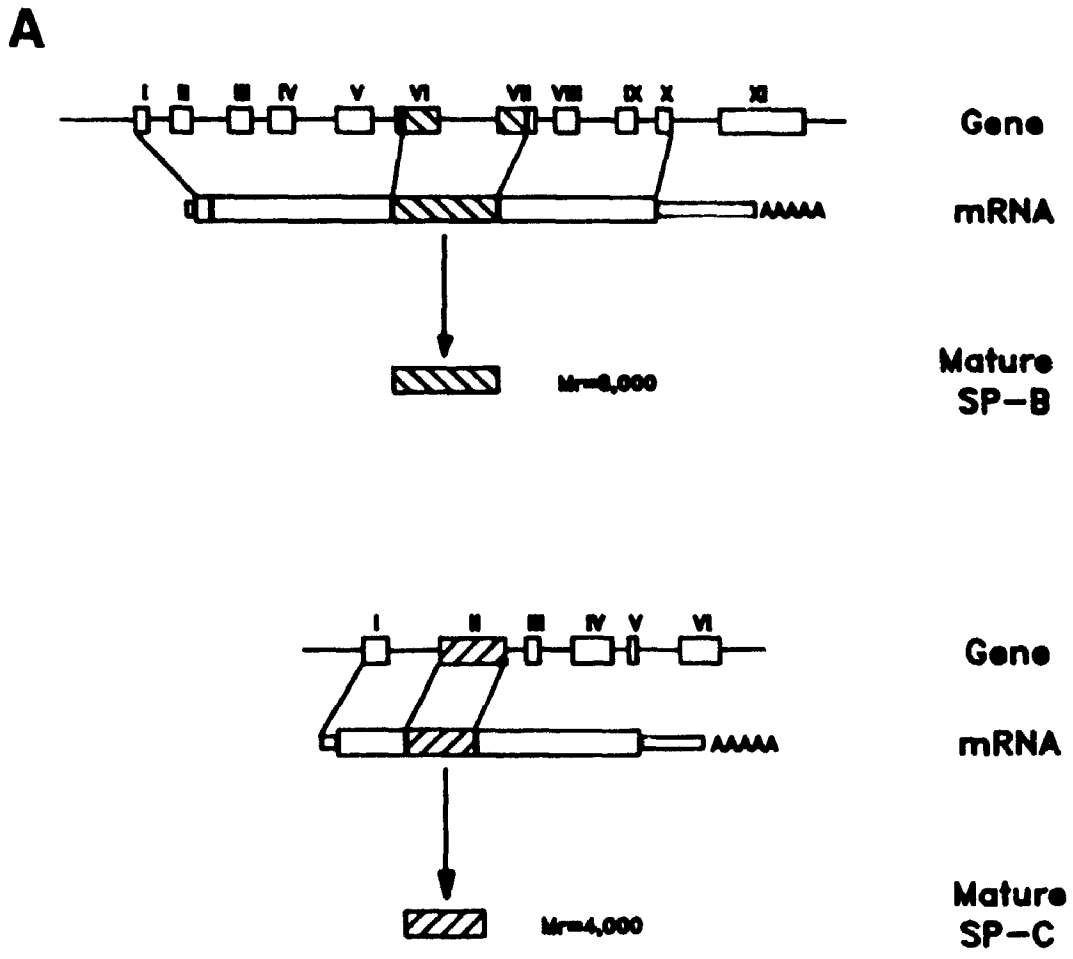
It has been suggested that the SP-B active peptide sequence contains three potential amphipathic helical regions, Leu_{10} -Pro_{32}, Leu_{57} - Pro_{67}, and Val_{31} - Val_{38} (Whitsett and Baatz, 1992). Together with the assignment of intramolecular disulfide

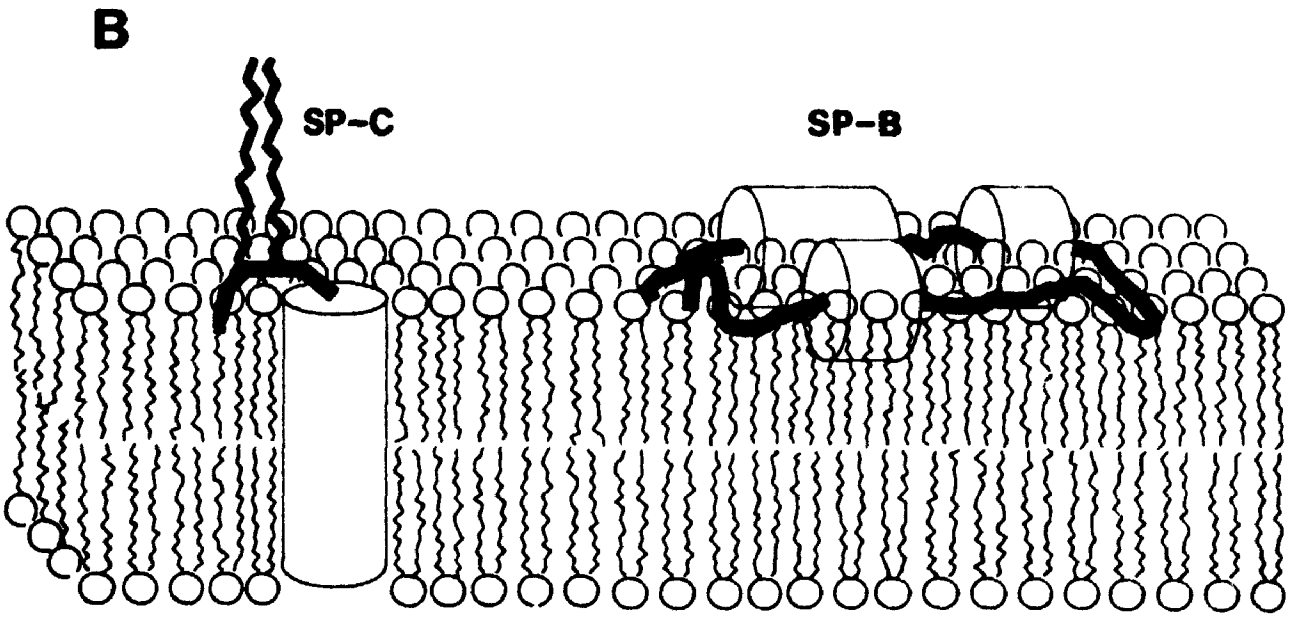
linkages in the bovine SP-B monomer (Johansson et al., 1991) (for detail see section 3.3), a theoretical model of SP-B in a membrane environment has been predicted and is shown in Fig. 1.4.(B) (Whitsett and Baatz, 1992).

Surface activity studies have demonstrated that SP-B has significant surface tension-lowering properties upon recombination with a mixture of DPPC and phosphatidylglycerol (PG) (Yu and Possmayer, 1988; Revak et al., 1988). In fact, SP-B containing mixtures can lower the surface tension of a pulsating bubble to near 0 mN/m in a manner similar to natural surfactant. This is supported by studies in intact animals which showed that instillation of phospholipid and SP-B into the trachea of fetal rabbits improved lung compliance and nearly normalized alveolar expansion (Revak et al., 1988). This surface active property of SP-B may be conferred by the two amphipathic helices proposed for the active peptide (Fig. 1.4.B), as indicated by studies using two synthetic peptides, one consisting of the N-terminal amphipathic helix and the other composing the C-terminal amphipathic region (Waring et al., 1989; Baatz et al., 1991). Studies with model membrane systems suggested that SP-B has a significant ordering effect at the membrane surface (i.e. in the phospholipid headgroup region) (Baatz et al., 1990) but little effect on the organization of the lipid acyl chains (Baatz et al., 1991). These results are consistent with the proposed presence of amphipathic helical structures in SP-B.

Other specific roles for SP-B in surfactant function include a specific interaction with the negatively charged PG head group (Baatz et al., 1991; Yu & Possmayer, 1990).

Figure 1.4. Gene and protein structures of SP-B and SP-C. (A) Schematic organizations of the SP-B and SP-C genes, mRNAs and airway peptides. The exons (boxes) and introns (lines) for the human genes are shown and numbered with Roman numerics. Coding sequences for the mature peptides of SP-B and SP-C are indicated by hatched areas. See Sections 1.1.4. and 1.3. for details in the sizes and structures of the mRNAs and proteins. (B) Schematic drawing of possible confirmations of SP-B and SP-C in a membrane bilayer. The three horizontal cylinders of SP-B correspond to the potential amphipathic helical regions noted in Section 1.1.1.4. and illustrated in Fig. 3.7. The single cylinder of SP-C represents the predicted membrane spanning helix (see text for details). The drawing is taken from Whitsett & Baatz (1992) with permission.


A
I II III IV V VI VII VIII IX X XII
Gene
mRNA
AAAAA
Mature
SP-B
Mr=8,000
I II III IV V VI
Gene
mRNA
AAAAA
Mature
SP-C
Mr=4,000


B
SP-C
SP-B

Yu and Possmayer in this laboratory have demonstrated that purified SP-B promotes phospholipid adsorption to form a phospholipid monolayer in the presence of calcium and that the protein may also act to enhance formation of DPPC-rich phospholipid monolayers by promoting squeeze-out of the acidic phospholipid (PG) from the monolayer upon compression (Yu and Possmayer, 1990, 1992).

In addition to the surface-active properties of SP-B, reconstitution experiments indicate that SP-B, along with DPPG, PG, SP-A and calcium, appears to be required for the formation of tubular myelin (Suzuki et al., 1989; Williams et al., 1991). SP-B and synthetic fragments of human SP-B enhance the uptake of phospholipids by type II cells in vitro (Rice et al., 1989) and therefore may play a role in the recycling of surfactant in the alveolus.

The second highly hydrophobic peptide, SP-C, is soluble only in organic solvents. This peptide can migrate as a dimer of ~7 kDa following SDS/PAGE under nonreducing conditions and is present as a monomer of ~3.5 kDa in the presence of reducing agents (for review see Possmayer, 1988). Amino acid sequence analysis of mature SP-C have identified three peptides of 33-35 residues differing only in the initiation of the N-terminus (Johansson et al., 1988). The sequence also revealed that SP-C has an unusual polyvaline region (6 consecutive residues) (Warr et al., 1987; Glasser et al., 1988). Two cysteine residues located near the N-terminus of SP-C molecule are palmitated in human, porcine and bovine SP-C (Curstedt et al., 1990). These two palmitoyl moieties are attached to proSP-C prior to its cleavage (Vorbroker et al., 1992). The SP-C proprotein or primary translation product is 197 amino acid long in the human with a molecular

weight of 21,000 (Warr et al., 1987; Glasser et al., 1988), which undergoes proteolytic processing to produce the 3.5 kDa mature protein found in bronchoalveolar lavage fluid and amniotic fluid. In contrast to SP-A and SP-B, proSP-C does not contain a classical leader sequence to target SP-C to the secretory pathway. In fact, SP-C shares structural similarities with known leader sequences, and therefore may be able to serve as a signal peptide. ProSP-C seems to be associated with subcellular membranes before undergoing further post-translational modifications (Vorbroker et al., 1992).

A theoretical model has been proposed for mature SP-C (Whitsett and Baatz, 1992) (Fig. 1.5.**B**). Experimental data, obtained using fourier transform infrared (Pastrana et al., 1991) and 2D-NMR demonstrated that SP-C is approx. 68% helical (Whitsett and Baatz, 1992). This corresponds to 23 amino acids from Lys^{11} to Leu^{35} (including the poly-valine region) being involved in α-helix formation as predicted from the sequence. This α-helix would have an approximate overall length of 36 Å. Therefore it suggests that SP-C could act as a membrane bilayer spanning protein (Fig.1.5.**B**). The N-terminal segment of the SP-C peptide segment from Cys^{5} to Lys^{11} is likely located near the bilayer surface. The two palmitoyl moieties attached to the adjacent cysteines near the N-terminal of the protein could be inserted into the same bilayer membrane probably resulting in stabilization of the membrane. This is supported by data from fluorescence experiments (Horowitz et al., 1992) which suggested that SP-C enhances the surface activity of surfactant by stabilizing the phospholipid membrane during compression and expansion.

The role of SP-C in the surface activity of surfactant is less obvious than that SP-

B or SP-A. SP-C enhances the adsorption of surfactant lipids (Yu & Possmayer, 1988; Hawgood et al., 1987). It also enhances the surface activities of synthetic lipid mixtures (Revak et al., 1988; Warr et al., 1987; Curstedt et al., 1990; Yu & Possmayer, 1988) although the activity was significantly lower than that observed with natural surfactant or SP-B/phospholipid preparations. In fact, SP-C and SP-B are the major protein constituents of organic solvent extracts of pulmonary surfactant which are effective in enhancing pulmonary functions when administrated to infants with NRDS (see section 1.1.1). In addition, recent studies also indicate that recombinant SP-C/phospholipids may be resistant to inhibition of surface activity by blood protein (Seeger et al., 1991; Venkitaramen et al., 1991).

Like SP-B, SP-C and SP-C peptide analogues enhance the uptake of phospholipids by type II cells (Rice et al., 1989), indicating SP-C also plays a role in surfactant recycling.

1.1.4.3. SP-D.

A fourth surfactant-associated protein, SP-D, has recently been identified (Persson et al., 1988, 1989). This protein, like SP-A, is a hydrophilic glycoprotein containing a collagenous sequence and a lectin domain. It is synthesized and secreted by alveolar type II cells and has been isolated from rat bronchoalveolar lavage fluid (Persson et al., 1988). In rat lung SP-D is synthesized as a 39 kDa primary translation product that undergoes post-translational N-linked glycosylation, as well as hydroxylation and glycosylation of lysine residues within the collagenous domain (Crouch et al., 1991b;

Shimizu et al., 1992). SP-D is secreted as a 43 kDa protein that can form a disulphide-bonded trimer. SP-D is similar to SP-A in that it contains a collagenase-sensitive domain, hydroxyprolines, a asparagine-linked oligosaccharide and lectin-like activity, but differs in that SP-D has different mobilities on polyacrylamide gel. In addition, SP-D is more basic than SP-A (pI 6-8 vs pI 4.5-5.0 of SP-A). Furthermore, the two proteins have different cyanogen bromide and proteolytic cleavage patterns (Persson et al., 1989). Immunodetection of SP-D and SP-A in fractions obtained by high-speed centrifugation of rat lavage indicated that a large portion of the SP-D was in the supernatant whereas almost all of the SP-A was in the pellet. This suggests SP-D may exist as a soluble protein in the alveoli (Kuroki & Akino, 1991c).

The structural similarities of SP-D and SP-A with respect to their collagenous structure and carbohydrate-binding capacity suggest that these two proteins may have similar functions. However, purified SP-D does not appear to contribute to the surface-active properties of surfactant phospholipids (Persson et al., 1990). In contrast to SP-A, SP-D does not bind tightly to lipids or cause lipid aggregation (Kuroki & Akino, 1991), does not appear to affect basal surfactant secretion or stimulate surfactant lipid secretion by type II cells, nor enhance uptake of phospholipid liposomes by these cells. Interestingly, native SP-D altered the inhibitory effect of SP-A on surfactant secretion (Kuroki et al., 1991a). Native SP-D was found to compete with [^{125}I]SP-A for high affinity binding sites on type II cells (Kuroki & Akino, 1991). The ability of SP-D to counteract SP-A's interaction with type II cells may be important physiologically.

1.1.5. Localization of expression of surfactant proteins

All of the surfactant-associated proteins identified to date, SP-A, B, C and D, are expressed in a tissue-specific manner. Their mRNAs and the protein synthesis are detected only in lung tissue.

Two cell types are known to express SP-A in lungs of several animal species. SP-A mRNA has been detected by in situ hybridization in both the alveolar type II cell and the non-ciliated bronchiolar cell or Clara cell (for review see Snyder 1991; Weaver and Whitsett, 1991). Antibodies against SP-A also detect this protein in both cell types (Walker et al., 1986; Williams et al., 1988). The presence of both SP-A protein and mRNA in Clara cells provides strong evidence that these cells express SP-A, although its physiological significance has not been defined. In adult human lung, SP-A mRNA is detected only in type II cells (Phelps & Floros, 1988), suggesting the possibility of species-specific expression of SP-A in the Clara cells.

The site of SP-A expression in developing lung has recently been examined in the rabbit (Wohlford-Lanene et al., 1992b). In this study, SP-A mRNA was first localized in alveolar type II cells on day 26 of gestation, the time at which lamellar bodies are first observed within fetal lung type II cells (Snyder & Magliato, 1991). On day 28 of gestation, a very small amount of SP-A mRNA was also detectable in the epithelial cells of some bronchioles. In neonatal and adult rabbit lung, SP-A mRNA was primarily localized in alveolar type II cells, however the epithelial cells of some bronchioles contained small amounts of SP-A mRNA.

The subcellular distribution of SP-A protein has been examined in lung tissue and

cultured type II cells. Studies using the immunoperoxidase technique showed that the rough endoplasmic reticulum, nuclear membrane, and Golgi complex of type II cells in adult rat lung tissue stain positively for SP-A (Williams et al., 1988). Kalina and co-worker detected SP-A immunoreactivity at the periphery of lamellar bodies in adult human lung tissue (Kalina et al., 1993). Studies in which SP-A was immunolocalized in cultured type II cells demonstrated that SP-A immunoreactivity was present in the endoplasmic reticulum, the Golgi complex, in small intracellular organelles, and on the surface of the cell (Liley et al., 1987a; Kalina et al., 1993).

SP-B is expressed in both type II cells and Clara cells in adult lung as demonstrated by in situ hybridization and immunostaining (for review see Snyder, 1991; Weaver and Whitsett, 1991). SP-B mRNA and protein are also detected in bronchiolar and alveolar epithelial cells and in tracheal glands in human (Phelps & Floros, 1988), rat (Phelps & Floros, 1991b), and mouse (D'Amore et al., 1992) lung. Examination of developing rabbit lung by in situ hybridization (Wohlford-Lanene et al., 1992b) revealed that SP-B mRNA could be detected in presumptive type II cells at least two days earlier than SP-A mRNA. As term approached, SP-B mRNA was also present in bronchiolar epithelial cells. The concentration of SP-B mRNA increased during development, which was also detected by Northern analysis (see section 1.2.2.2). This increase is due to elevations in both type II cells and bronchiolar cells (Wohlford et al., 1992b). In adult rabbit lung, the concentration of SP-A was present in approximately equal amounts in the two cell types (Wohlford et al., 1992b). Within type II cells, SP-B was localized to the core of lamellar body, which is consistent with its hydrophobic nature.

Similar to SP-A and SP-B, SP-C is also expressed in a cell-specific manner. However, SP-C mRNA is readily detected by in situ hybridization very early in fetal life, as early as day 11 of mouse gestation (term 22 days) (Glasser et al., 1990). SP-C mRNA is present in all epithelial cells in the early embryonic lung but gradually become restricted to differentiating type II cells close to term and is confined to type II cells in the adult murine and rabbit lung (Wohlford et al., 1992a; Whitsett and Baatz, 1992).

Immunohistochemical studies using antibodies against SP-D showed no staining in fetal rat lung on days 17 or 19 but strong cytoplasmic staining of cuboidal epithelial cells (see section 1.2.1.2) on day 21, one day before term (Crouch et al., 1991a). In adult rat lung, SP-D protein was detected in type II cells, Clara cells and alveolar macrophages (Voorhout et al., 1992). Lamellar bodies did not appear to contain SP-D (Voorhout et al., 1992).

1.2. Lung Maturation and Developmental Regulation of the Surfactant System

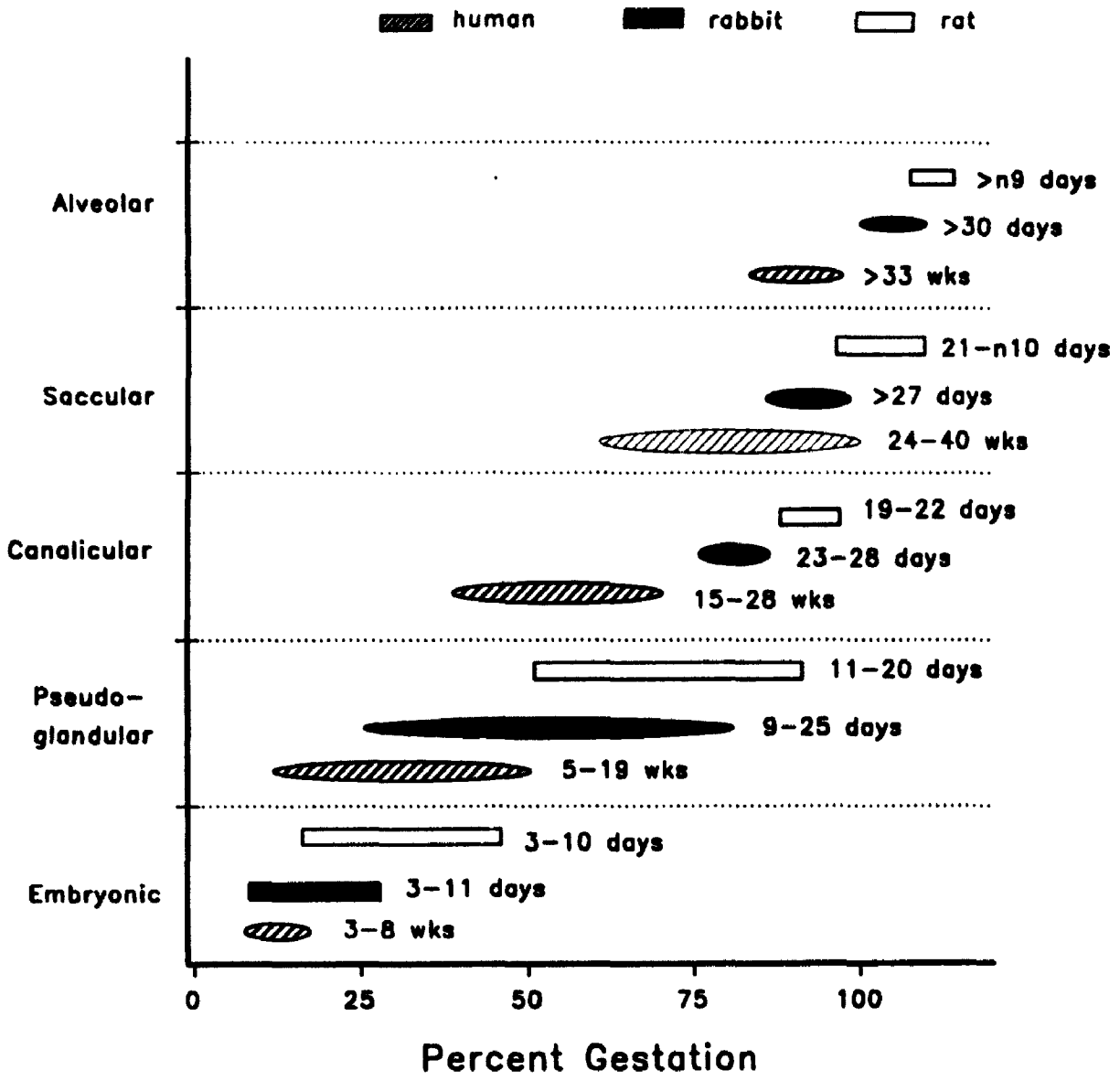
1.2.1. Embryology and morphogenesis of the lung

1.2.1.1. Embryology of the lung

Lung development can be divided morphologically into five phases: embryonic, pseudoglandular, canalicular, saccular and alveolar phases (Fig.1.5) (Pringle, 1986; Bourbon, 1991).

(1) The embryonic phase: The lung originates from two distinct tissue layers, the endoderm and the mesoderm. The primordium of the respiratory system arises as an endodermal outgrowth of the foregut which extends into the surrounding mesoderm. The

Figure 1.5. Comparison of the morphological development of the human, rabbit and rat lungs. For each species (human, hatched bars; rabbit, solid bars; rat, open bars), duration of each of five phases of lung development (Y-axis) is shown in proportion of gestation (X-axis) (n: neonatal). Note that there is considerable variation between the three species in the proportion of gestation occupied by the various phases of lung development. This species variation is most marked in the pseudoglandular and alveolar phases of development. Diagram modified from Pringle, 1986.


human
rabbit
rat
Alveolar
>n9 days
>30 days
>33 wks
Saccular
21–n10 days
>27 days
24–40 wks
Canalicular
19–22 days
23–28 days
15–28 wks
Pseudo–
glandular
11–20 days
9–25 days
5–19 wks
Embryonic
3–10 days
3–11 days
3–8 wks
0
25
50
75
100
Percent Gestation

lung primordium develops into a recognizable "lung bud", then forms the early precursors of the lung lobes. The tracheobronchial tree is formed by sequential branchings.

(2) The pseudoglandular phase: By the beginning of fetal life (the 8th week in the human; term 40 weeks), the lung is clearly in the pseudoglandular phase. This phase is characterized by smooth-walled, blindly ending respiratory channels lined by cuboidal epithelium and separated by relatively thick, sparsely cellular interstitial septa. In the rabbit, lung of gestational day 21 (term 31 days) is in the glandular stage (Kikkawa et al., 1971).

(3) The canalicular phase: This phase begins with a thinning of the epithelium and the development of a more irregular configuration of the airways. The canalicular phase reflects the beginning of vascularization of the respiratory system. In the human, type II cells develop during the later stages of this phase. Rabbit lung starts to show the initiation of this stage on gestational day 24. It extends until day 26 when many alveolar epithelial cells remain cuboidal (Kikkawa et al., 1971).

(4) The saccular phase: This phase is marked by completion of vascularization of the respiratory surface. It begins in rabbit lung around day 27 of gestation, with not so-well recognizable epithelial cells appearing in large portions of the lung. This phase extends into the postnatal period in rabbit lung, overlapping with the next phase of lung development.

(5) The alveolar phase: This stage can begin as early as 30 weeks in some human fetuses and is clearly present by 36 weeks (Langston et al., 1984). During the period

of 32-36 weeks in the human, the type II cells mature, completing the functional maturity of the lung. However, the bulk of the alveoli are formed after birth, and these continue to develop up to eight years of age.

In the human lung, the pseudoglandular phase is virtually complete by 50% gestation, with the saccular and alveolar phases beginning at 60% and 75% of gestation, respectively. In contrast, the pseudoglandular phase of the rabbit lung extends to 23-24 days' gestation (out of 31 days for a normal pregnancy), occupies 75% of gestation, and the three remaining phases proceed rapidly at a rate of 3-4 days each (Fig. 1.5).

1.2.1.2. Cytodifferentiation of type II cells

During the embryonic phase of lung development, the walls of the lung primordium are lined with undifferentiated columnar epithelial cells. At the pseudoglandular phase, the undifferentiated cells start to differentiate into prospective airway (bronchiolar) epithelium and prospective respiratory (alveolar) epithelium. The epithelial cells in pre-alveolar regions change from tall columnar to cuboidal which then begin expressing certain phenotypic features of type II cells (Funkhouser et al., 1987). At this stage of development the cells do not contain lamellar bodies and can be called protodifferentiated type II cells. The protodifferentiated type II cells, which have a high glycogen content (Carlson et al., 1987) (see below), proliferate rapidly during the budding of the airways. At the later stages of the pseudoglandular phase, proliferation slows and the protodifferentiated type II cells start to mature further as the number and size of lamellar bodies increase and the glycogen content decreases (Carlson et al.,

1987). Maturation of type II cells during the canalicular phase of lung development is further marked by an expanding endoplasmic reticulum and an increased number of multivesicular bodies which are thought to participate in lamellar body formation (Carlson et al., 1987).

In rabbit lung, tall columnar epithelium is observed on day 21 of gestation. The apical cytoplasm contains a moderate amount of scattered glycogen. By day 24, most of the epithelium is either shortened columnar or cuboidal. The glycogen zone within the epithelium is expanded, and the glycogen granules are more tightly packed compared to those in the lung of the 21-day-old fetus. A few inclusion bodies are found in approximately 7% of the epithelia cells in the lung (Kikkawa et al., 1971; Wang et al., 1971). In the lung of rabbits at day 26, type II cells are well differentiated. The type II cell, which can be readily recognized by the presence of well-formed lamellar bodies, is cuboidal, and still contains a large amount of glycogen (Kikkawa et al., 1971). The type II cells account for about 23% of total epithelial cells in rabbit lung of this age (Wang et al., 1971). On day 28 of gestation, complete maturation of the type II cell can be observed by electron microscopy. There are numerous lamellar bodies and the loss of glycogen continues (Kikkawa et al., 1971). Approximately 39% of the epithelial cells are type II pneumonocytes on day 28 (Wang et al., 1971).

1.2.2. Developmental profiles of the surfactant system

1.2.2.1. Lipids and glycogen

Glycogen, detected in committed alveolar epithelium in the early stages of

development, may provide both energy and substrates for the synthesis of the surfactant phospholipids (Farrell & Bourbon, 1986). The glucose produced by glycogenolysis is metabolized to glycerol-3-phosphate, which can be incorporated into the glycerol backbone of phospholipids. Glycogen also can provide substrates for fatty acid synthesis. It is well established that in many species, the glycogen content of the lung increases during early gestation and declines rapidly during the period of maximal surfactant production late in gestation (Cockshutt and Possmayer, 1992).

Depending on the species, the relative amount of phosphatidylcholine rises from approx. 40% of the total phospholipids in immature lung to 60% at term (for review see Cockshutt and Possmayer, 1992). The elevation in phosphatidylcholine (PC) is accompanied by a decrease in sphingomyelin (SM) resulting in a marked increase in the lecithin (phosphatidylcholine) to SM (L/S) ratio during development (Rooney, 1986). The changes in L/S ratio in lung are also apparent in amniotic fluid and therefore the L/S ratio in amniotic fluid is widely used to assess lung maturity of the human fetus (Hallman 1984). It has been reported that in fetal rabbit lung, phosphatidylcholine content increases three-fold from day 28 of gestation to term (for review see Ballard et al., 1992).

The enhanced phosphatidylcholine synthesis is accompanied by a number of changes in the activities of the enzymes associated with the biosynthesis of phosphatidylcholine. CTP:phosphocholine cytidylyltransferase (CPCT) has been postulated to play a rate-regulatory role in phosphatidylcholine biosynthesis. The activity of CPCT starts to increase shortly before birth (Post & van Golde, 1988). Carlson and

coworkers (Carlson et al., 1988) have demonstrated that the specific activity of CPCT increases in rat type II cells during perinatal lung development.

Near term the composition of acidic phospholipids in lung lavage and lamellar bodies alters in most mammalian species. At this time, the relative amount of phosphatidylglycerol increases whereas that of PI decreases. In human amniotic fluid the percentage of PI declines at 35 weeks gestation concomitantly with a rise in the percentage of phosphatidylglycerol. This has enabled its use as an important second estimator of lung maturity from amniotic fluid samples (for review see Hallman, 1984).

1.2.2.2. Surfactant proteins

It has been known that production of surfactant proteins is regulated developmentally. The proteins are not detected until late in gestation. Although expression of the mRNAs for these proteins appears coordinated towards term (Connelly et al., 1991), each surfactant protein gene is controlled by different mechanisms.

(a) SP-A: The developmental expression of SP-A was first evaluated using an ELISA to measure SP-A contents in rat fetal lung and amniotic fluid (Katyal and Singh, 1983). In this study, SP-A was first detected on day 18 of gestation (term 22 days) in fetal lung tissue and on day 19 in amniotic fluid. Fetal lung SP-A content increased during the remainder of gestation and increased further in neonatal lung tissue. SP-A content was highest in adult rat lung tissue. This developmental pattern of SP-A protein accumulation in rat lung tissue was recently confirmed by Shimizu et al. (1991). Schellhase et al.

reported a similar developmental pattern of SP-A mRNA in fetal rat lung tissue (Schellhase et al., 1989).

Data on developmental SP-A expression in human fetal lung are available only during the early gestational period. SP-A mRNA was low or undetectable in fetal lung tissues obtained between 15 and 24 weeks of gestation by immunoblot analysis (Ballard et al., 1986) and detected at very low levels in human fetal lung tissue obtained between 20 and 24 weeks of gestation by use of an ELISA (Whitsett et al., 1987a). SP-A was first detected in human amniotic fluid from ~30 week gestation (Snyder et al., 1988) and continued to increase throughout the remainder of gestation in association with an increase in the levels of surfactant phosphatidylglycerol (PG) (Snyder et al., 1988; Kuroki et al., 1985).

In the rabbit, SP-A mRNA was first detected on day 24 or 26 of gestation (term 31 days) and increased in concentration thereafter until birth (Boggaram et al., 1988a; Connelly et al., 1991; Durham et al., 1993). An identical pattern of SP-A gene transcription has been observed (Boggaram et al., 1988b). SP-A content in fetal rabbit lung follows a similar developmental pattern as SP-A mRNA (Snyder and Mendelson, 1987a). Unlike the finding reported for the rat, SP-A mRNA concentration in adult rabbit lung tissue was found to be less than that in late fetal and neonatal lung tissue (Boggaram et al., 1988; Connelly et al., 1991). During gestation there are changes in the apparent molecular weight of SP-A protein. In lung homogenates from 24-28 day gestational age fetal rabbits, immunoreactive SP-A is present as a 29,000 M_r species, pI < 5.6, whereas, in lung homogenate from 30 day fetal rabbit, neonates and adults, the

major immunoreactive species is the fully glycosylated, 29-36,000 M_r form of the protein (Snyder & Mendelson, 1987a). Only the fully glycosylated form of SP-A is detectable in lamellar bodies, the intracellular pool of surfactant, isolated from lung tissue of 28-31 day fetal rabbits, neonates and adults (Snyder & Mendelson, 1987).

(b) SP-B and SP-C: In human fetal lung tissue, initiation of expression of the genes encoding SP-B and SP-C appears at a much earlier time in development than is the case for SP-A. The mRNAs for SP-B and SP-C are detectable in human fetal lung as early as 13 or 16 weeks of gestation (Liley et al., 1989; Whitsett et al., 1987a) and continue to increase thereafter until 24 weeks (Liley et al., 1989). SP-A mRNA is not detected within this period (Liley et al., 1989).

In rats, it was observed that SP-B mRNA was first detected in fetal lung tissue on day 18 of gestation (as is the mRNA for SP-A), whereas the mRNA for SP-C was readily detectable as early as day 17. Both mRNA species increased until term (Schellhase et al., 1989). In contrast to SP-A mRNA, which only reaches adult levels by postnatal day 28, the mRNAs for SP-B and SP-C were found to attain adult levels by day 20 of gestation (Schellhase et al., 1989). Immunoreactive SP-B and proSP-C are detectable in day 17 rat lung homogenate, increase gradually during gestation and show a marked increase after birth, the maximum values being attained on day 4 of neonatal life (Shimizu et al., 1991).

The developmental profiles of SP-B, SP-C, along with SP-A mRNA in rabbit fetal lung have recently been examined by Connelly in this laboratory (1991) and Durham et

al. (1993). Although there are differences in the times SP-B mRNA is first detected by these two groups of investigators (see chapter 4 for detailed discussion), it is clearly shown that SP-B and SP-C mRNA are apparent in fetal rabbit lung before the detection of SP-A mRNA and continue to increase until birth. This is consistent with the developmental profiles of the mRNAs for these three proteins obtained in other species as noted above.

(c) SP-D: SP-D mRNA and immunoreactive SP-D protein are first detected in rat lung of day 21 gestation and reach even higher concentrations during the postnatal period (5 days) (Crouch et al., 1991a). The increase in total lung SP-D and SP-D mRNA are temporally correlated with SP-D secretion and the appearance of SP-D in amniotic fluid (Crouch et al., 1991a). It appears that SP-D protein is synthesized in the rat lung later than the other three surfactant proteins. Data on the changes of SP-D mRNA and protein during lung development in other species has not been available.

1.3. Genetic Structures of the Genes Encoding for SP-A, SP-B and SP-C

1.3.1. SP A gene.

The SP-A gene has been characterized in the human (White et al., 1985) and rabbit (Chen et al., 1992). The human SP-A gene is mapped to the long arm of chromosome 10 (Bruns et al., 1987; Fisher et al., 1987). In both species, the gene is approx. 5 kb in length and consists of five exons and four introns (Fig. 1.3. A). The SP-A coding sequence is distributed among exons 2-5. The 5' untranslated region

consists of less than 100 nucleotides on exon 1 and 2. The relatively long 3' untranslated region (~ 1,300 nucleotides) is encoded by exon 5. There are sequences with homology to the glucocorticoid receptor elements (GRE) that have been characterized in other eukaryotic genes (for review see Latchman, 1991). In the human gene, one potential GRE is found ~ 100 bp upstream from the transcription initiation site (White et al., 1985). Rabbit SP-A gene contains four half-site putative GREs, two residing in the 5' flanking sequence and two within the first intron. In addition, one region homologous to cyclic AMP responsive elements (CRE) is found in the rabbit gene upstream of the transcription initiation site (Chen et al., 1992). The presence of these regulatory elements could suggest that SP-A expression is directly regulated by glucocorticoids and cyclic AMP. A lung-specific DNase I hypersensitive site has also been identified in the rabbit SP-A gene (Chen et al., 1992). This site (-80 to -180) is spatially close to two of the GREs identified in the rabbit gene (-150, -190). Whether this tissue-specific site is related to glucocorticoid regulation has not been investigated. This DNase I hypersensitive site needs to be further defined.

It has been suggested by restriction mapping studies that there is more than one SP-A gene in the human (Fisher et al., 1987). A human SP-A pseudogene has been identified. It contains a sequence with 84% homology to intron 4 and exon 5 of the human SP-A gene and maps to chromosome 10 as well (Korfhagen et al., 1988). Two similar but distinct human SP-A cDNAs (termed 1A and 6A) have been reported (Floros et al., 1986). The available human SP-A gene sequence (White et al., 1985) shows homology to the 6A cDNA. The 1A gene has not been identified and its relationship

with the SP-A pseudogene has not been established. In contrast, studies in rat (Fisher et al., 1988b), rabbit (Boggaram et al., 1988a, 1988b) and mouse (Korfhagen et al., 1990) suggest that there is only one SP-A gene in these species. The functional implication of multiple human SP-A genes remains unclear.

1.3.2. SP-B gene.

The human SP-B gene has been characterized as a sequence spanning approx. 9.5 kb with 11 exons and 10 introns and is located on chromosome 2 (Pilot-Matias et al., 1989, Fig. 1.4. A). Restriction mapping of human and rat genomic DNA is consistent with a single SP-B gene (Pilot-Matias et al., 1989; Emrie et al., 1988). The very short 5' untranslated region of 14 nucleotides, determined by direct RNA sequencing (Pilot-Matias et al., 1989) of the human SP-B mRNA, is encoded on exon 1 while exon 11 encodes 823 bp of 3' untranslated region (Pilot-Matias et al., 1989). Within the 5' flanking region of the SP-B gene, one putative CRE and several GREs have been found (Pilot-Matias et al., 1989), which again imply direct regulation of this protein by glucocorticoid and cyclic AMP.

A partially characterized murine SP-B gene (D'Amore et al., 1992) shows a similar spatial arrangement of the promoter and exon 1 region, predicting a short 5'-UTR, as observed in human SP-B gene. The rabbit SP-B gene has not been characterized.

1.3.3. SP-C gene.

The human SP-C gene has been localized to the short arm of chromosome 8 (Fisher et al., 1988; Glasser et al., 1988). The gene is organized into six exons spanning approx. 2.7 kb (Glasser et al., 1990) (Fig. 1.4. A). The 25 basepairs of 5'noncoding region is encoded on exon 1 and the 3' untranslated region on part of exon 5 and all of exon 6. Two closely related SP-C genes, both of which are transcribed, are present in the human genome (Glasser et al., 1990). Nucleotide differences are restricted to exon 6 and the introns and flanking regions. The organization of the mouse SP-C gene is similar to that of the human but contains an insertion of 533 basepairs within intron 1 (Glasser et al., 1990). Two putative CREs are present in the 5'-flanking region of the gene, within 500 bp of the site of transcription initiation. No potential GRE has been identified within this region (Floros et al., 1986). The rabbit SP-C gene has not been characterized.

1.4. Regulation of Gene Expression of Surfactant Proteins

Gene expression of a specific protein can be regulated at many different levels, such as at the levels of initiation, elongation and termination of transcription, at the level of mRNA stability, of mRNA processing, at the level of translational efficiency, and protein processing and compartmentalization. Among these the role of transcriptional initiation has been emphasized as a decisive factor. The core promoter elements, including TATA-box, CAAT-box and GC-box, are required for basal level gene transcription. Enhancer sequence elements, e.g. hormone responsive elements (e.g. GRE

or CRE), modulate the basal transcriptional activity of the target gene resulting in activation or inactivation of gene activity. Another important component of gene regulation is mRNA turnover. Alterations in mRNA stability provide a rapid means of increasing or decreasing the pool of translatable mRNA and ultimately the amounts of protein. Thus, differential turnover rates for different mRNAs can permit rapid changes in the pattern of cellular gene expression and can allow for the economical maintenance of a translation template.

1.4.1. Some aspects of transcriptional and post-transcriptional regulation.

1.4.1.1. Transcriptional regulation

According to current views, two components are involved in transcriptional regulation: *cis*-acting elements or sequences and *trans*-acting factors. One of the first systems in which transcriptional regulation was studied in terms of regulatory sequences and the corresponding *trans*-acting factors was that of steroid-inducible gene expression (Latchman, 1991). The steroid hormones act by interacting with specific receptor proteins (Beato, 1989). The binding of hormone to its receptor activates the receptor and allows it to bind to a specific site in chromatin (e.g. GRE). In turn this DNA binding activates transcription of genes carrying the receptor binding site. These receptor proteins are transcription factors which become activated in response to a specific signal and in turn activate or inhibit specific genes (for review see Beato, 1989; Latchman, 1991).

Genes which are induced by steroid hormones contain similar but distinct binding

sites for the receptor-hormone complexes activated by different hormones in the steroid hormone family. The response to a particular steroid hormone is mediated by a specific binding site for the respective steroid hormone (Beato, 1989). Also, each steroid hormone is recognized by a different member of a family of homologous receptor proteins. The results of cDNA cloning, and comparison of the deduced amino acid sequences of different hormone receptors (for review see Beato, 1989) have shown that all receptors are structured in a similar way: a variable N-terminal region, a short, cysteine-rich central domain, and a relatively well-conserved C-terminal half. These three main domains are separated by less well-defined regions. The central domain is the most conserved region between different receptors and is responsible for the DNA binding activity of the receptors (Evans, 1988). The cysteine residues contained in this region are able to form two so-called zinc fingers (Evans, 1988). Both DNase I protection and methylation studies support the idea that the receptor binds to DNA as a dimer, each receptor molecule binding to one half of a palindromic recognition sequence (Beato, 1989). The N-terminal region appears to be involved in intranuclear localization of the glucocorticoid receptor (Beato, 1989).

It has been postulated that hormonal activation of steroid receptors is a multiple process. From a detailed study with the human progesterone receptor (PR), Edwards and co-workers (1991) showed that receptors in the absence of hormone are bound with hsp90 (heat shock protein 90), and perhaps with other proteins (hsp70) to form an inactivated complex. Interaction with hsp90 represses DNA-binding activity, rendering receptors unavailable for gene transcription until needed (Latchman, 1991). Receptor

activation, either in vitro or in intact cells, results in a loss of association with hsp90 (Latchman, 1991). This is followed by dimerization of PR polypeptides to form 6S dimeric molecules as an intermediate step prior to DNA binding (Edwards et al., 1991). In a number of cases, steroid hormone treatment has been shown to cause the induction of a DNase I hypersensitive site located at the DNA sequence to which the receptor binds (Latchman, 1991). Hence the binding of the receptor may activate transcription by displacing a nucleosome from the promoter of the gene, creating the hypersensitive site. In turn it would facilitate the binding of other transcription factors necessary for gene activation. It has also been suggested that the steroid receptors contain specific activation domains which can activate transcription when linked to the DNA-binding regions of other factors (for review: Latchman, 1991). Thus steroid receptors as activated *trans*-acting factors promote transcription both by altering chromatin structure after binding to their cognate *cis*-acting sequences to allow constitution factors to bind and also by interacting directly with those other factors (Latchman, 1991).

1.4.1.2. Regulation of mRNA stability

There are several classes of mRNA exist in a eukaryotic cell. The first class are highly unstable mRNAs which often encode regulatory polypeptides such as transcription factors E1a, E1b or oncogenes c-myc, c-fos (for review see Ragshow, 1987). This temporary nature enables the rapid down-regulation of protein synthesis shortly after the transcription of their mRNAs has ceased. The half-lives of these mRNA are in the range of 30 min. Those mRNAs whose translation products are required in relatively constant

amounts, e.g. "house-keeping" proteins, tend to be more stable. The half-lives of these mRNAs span from several hours to days (Ragshow 1987). Differential turnover rates for different mRNAs imply selectivity in regulating the degradation of certain mRNA species. However, little has been learned as to which ribonucleases are responsible for constitutive mRNA turnover, which signals on the mRNA they recognize or alter, and along which pathway(s) mRNA degradation occurs.

Some structural features commonly present in eukaryotic mRNAs have been suggested to be the signals which may trigger the mRNA degradation machinery. The 5' 7-methylguanosine triphosphate cap structure and the 3' poly (A) tail may stabilize mRNAs against degradation (Bernstein & Ross, 1989a, 1989b; Jackson & Standart, 1990). It has been noticed that the rate of mRNA turnover is highly dependent on the presence of the 3' terminal poly(A) sequences since deadenylated mRNAs having much shorter half-lives than adenylated controls as observed with globin mRNA & histone mRNA (Brawerman, 1987). The extremely short-lived c-fos and c-myc mRNAs show very rapid poly(A) shortening prior to its degradation (Brewer & Ross, 1988; Swartwout & Kinniburgh, 1989; Wilson & Treisman, 1988). The mechanisms involved have not been completely established. It is likely that some cytoplasmic binding proteins complex with polyadenylated RNA to form ribonuclear-protein particles. The best characterized of these proteins is the Poly(A)-Binding Protein (PABP). It is likely that PABP associated with the poly(A) tail protects mRNAs from rapid nucleolytic attack (Bernstein & Ross, 1989).

Elements unique to specific mRNAs have been identified. Such specific mRNA

elements are thought to interact with cytoplasmic *trans*-acting factors. One of these elements is the (AUUUA) motif observed in the 3' untranslated regions (UTRs) of cytokine mRNAs (Shaw & Kaman 1986), as well as of oncogenes and of some transcription factor mRNAs (Brawerman, 1989; Shaw & Kamen, 1986). Inclusion of the AUUUA motif targets mRNAs for rapid cytosolic degradation (Shaw & Kamen, 1986; Rahmsdorf et al., 1987; Wilson & Treisman, 1988). Recently, several AU-specific mRNA binding proteins have also been identified (Brewer, 1991). It is postulated that these proteins participate in the regulation of labile mRNA turnover through an interaction with the AU-rich domain. The potential for protein-protein interactions among these factors as well as their relative affinities for the AU-rich region may determine the stability of the mRNA. The origin of these proteins, however, has not been defined. It would be interesting to know how the activities of these proteins would relate to or respond to different conditions occurring in the cell.

Other specific structural elements are (1) a highly conserved 3' terminal stem-loop found in histone mRNA (Capasso et al., 1987; Graves et al., 1987); (2) the conserved structures found in the 3' untranslated region of the transferrin receptor mRNA (IRE, iron-responsive elements), which are composed of a stem-loop and a palindromic repeat. These structures render the regulation of respective mRNA stability in a cell cycle or iron-dependent manner (Marzluff & Pandey, 1988; Casey et al., 1989; Mullner & Kuhn, 1988). Selective removal of these structures abolishes normal regulation of the mRNA stability and varies the half-lives in a manner independent of the cell cycle or non-iron responsive (Capasso et al., 1987; Graves et al., 1987; Atwater, 1990). A cytoplasmic

protein that specifically recognizes and binds the IRE (IRE binding protein) has been identified (Haile et al., 1989; Leibold & Munro, 1988). Binding of the IRE binding protein to the 3' untranslated region IRE inhibits degradation of the normally labile TfR mRNA (Casey et al., 1989; Mullner & Kuhn, 1988).

It is now well established that the degradation of certain mRNAs is tightly coupled to their translation. Cycloheximide (CHX), an inhibitor of polypeptide chain elongation, causes stabilization of many unstable mRNAs (Taniguchi, 1988). Usually, when protein synthesis is blocked with CHX followed by an actinomycin D chase, a 4- to 8-fold inhibition of mRNA decay is observed. Three possible explanations have been postulated (Atwater, 1990): (1) nucleases or other proteins involved in mRNA degradation are polysome-associated; (2) a component of the degradation machinery is extremely labile and is rapidly cleared upon the cessation of protein synthesis and (3) mRNA turnover is autoregulated by the nascent peptide as it emerges from the polysome.

Steroid hormones are also determinants of mRNA stability (Nielson & Shapiro, 1990; Shapiro, 1987). In particular, human growth hormone (Diamond & Goodman, 1985; Paek & Axel, 1987), vasopressin (Atwater, 1990), a very low density apolipoprotein (Cochrane & Deeley, 1988) and insulin mRNAs (Muschel et al., 1986) are stabilized by hormones. Generally, the poly(A) tails of these mRNAs are lengthened which may directly account for their enhanced stability. In estrogen-treated Xenopus liver cells, vitellogenin mRNA increases approx. 50-fold (Atwater, 1990; Nielson & Shapiro, 1990) with an increase in the half-life from 16 hours to about 500 hours in the presence of estrogen (Atwater, 1990). The mechanisms involved are not clear. It has

been hypothesized that the estradiol-estrogen receptor complex may induce transcription of a gene(s) coding for a protein(s) which mediates stabilization (Nielson & Shapiro, 1990).

1.4.2. Regulation of surfactant protein gene expression

Fetal lung maturation is influenced by a variety of factors including cell-to-cell interactions (Post, 1992) and the action of local paracrine mediators and circulating hormones (for review see Gross, 1990; Mendelson & Boggaram, 1991; Snyder, 1991; Whitsett & Weaver, 1991). Hormones appear to influence the later phases of fetal lung development. Numerous studies have been conducted to investigate the glucocorticoid regulation of fetal lung cell morphology and the functional development of the surfactant system, including the surfactant phospholipid synthesis and surfactant associated protein synthesis. In addition, thyroid hormone, epidermal growth factor (EGF) and adenosine 3',5'-cyclic monophosphate (cAMP) enhance a number of components of lung maturation in vivo and in vitro (for reviews: Gross, 1990; Weaver and Whitsett, 1991; Post, 1992). There are also agents that inhibit fetal lung maturation. These include maternal diabetes, male sex, which may be related to an androgen effect on surfactant phospholipid synthesis; and transforming growth factor-beta (TGF-β) (see below).

Most in vitro studies concerning the hormonal regulation of surfactant proteins have been performed using undifferentiated fetal lung explants as a model system (Funkhouser , 1976), since isolated type II pneumonocytes are difficult to maintain in a differentiated state in vitro (Liley et al., 1988a; Weaver et al., 1986). The epithelial

cells in these explants from fetal rat, rabbit, mouse or human lungs develop the capacity for surfactant phospholipid synthesis in culture (Mendelson et al., 1986; Gross et al., 1989a). These epithelial cells also contain morphologically identifiable lamellar bodies and measurable SP-A protein and mRNA. This spontaneous differentiation of lung explants in vitro in a serum-free environment makes the system a very useful model for studying the regulation of type II cell differentiation and regulation of surfactant protein gene expression by defined circulating hormones or agents. Another advantage offered by the explant system over primary culture of purified type II cells is that fetal lung epithelial cells within the explants retain the three-dimensional tissue environment and cell-cell interactions which appear to be crucial for the maintenance of type II cell differentiation (Liley et al., 1988a; Weaver et al., 1986).

Several cell lines have been established from human lung adenocarcinomas. These are basically used for studying the regulation of surfactant protein expression. One of the cell lines H-820 (O'Reilly et al., 1989b) has morphological features compatible with a close phenotypic relationship to type II cells and can produce the three surfactant proteins, SP-A, SP-B and SP-C. Another cell line H-441 (O'Reilly et al., 1988) contains lipoidal inclusions and dense cytoplasmic granulates that are similar to those seen in Clara cells. It has no morphological features of type II cells. This cell line expresses SP-A and SP-B, but not SP-C.

In addition, several studies have been carried out with in vivo systems. The levels of the surfactant proteins and their mRNAs are measured in fetal lung tissue after maternal administration with different hormones for various periods of time (see below).

1.4.2.1. Glucocorticoid regulation

(a) SP-A:

Glucocorticoids have complex actions on SP-A gene expression in fetal lung tissues. In human fetal lung culture, glucocorticoids can stimulate as well as inhibit production of SP-A (Liley et al., 1987, 1988b; Ballard et al., 1986a; Whitsett et al., 1987a; Odom et al., 1988). This biphasic response to glucocorticoid is also reflected at the level of SP-A mRNA production (Liley et al., 1988b; Odom et al., 1988). In time course studies, SP-A mRNA is detected after 2 days in control cultures and increases to levels comparable to those in adult lung. The presence of 1 μM cortisol initially accelerates the appearance of SP-A (days 2 and 3) and later inhibits accumulation of message. When hormone is removed from the treated cultures, both the stimulatory (Liley et al., 1988b) and the inhibitory effects are reversed. Similar responses are seen with both synthetic and natural glucocorticoid and in a human lung adenocarcinoma cell line (O'Reilly et al., 1989b). In dose-response studies, with dexamethasone at concentrations of 10^{-10} and 10^{-9} M, a stimulatory effect is observed, while at concentration over 10^{-8} M, the glucocorticoid was markedly inhibitory (Odom et al., 1988). This is not due to changes at the transcriptional level, since dexamethasone exerts only stimulatory effects on SP-A gene transcription which are dose- and time-dependent (Boggaram et al., 1989). Further studies on the mechanisms underlying the biphasic effects of glucocorticoid on SP-A mRNA levels in human fetal lung tissue in vitro (Boggaram et al., 1991) revealed that: (1) Dexamethasone (10^{-7} M) has no adverse effect on the elongation of nascent mRNA transcripts throughout the SP-A gene, ruling out the

possibility that there was an increase in transcription with premature transcription leading to instability of mRNA transcripts; (2) Dexamethasone (10 M) has a pronounced effect on reducing the apparent half-life of SP-A mRNA. In control explants maintained in the presence of actinomycin D to abolish gene transcription, the SP-A mRNA half-life was estimated to be 11.4 h, whereas in tissues also treated with dexamethasone, the SP-A mRNA half-life was reduced by more than 60% to 5.0 h; (3) This effect of dexamethasone on the degradation of SP-A mRNA is dose-dependent, with higher doses of the hormone having more pronounced effects on reducing SP-A mRNA levels (Boggaram et al., 1991).

Studies in fetal rabbit lung by Mendelson and colleagues (Mendelson et al., 1986; Boggaram & Mendelson, 1988b) provide additional evidence for the complexity of the glucocorticoid effects on SP-A. Treatment of explants with cortisol (10^7 M) decreases the content of SP-A mRNA after 12 h; however, accumulation of the message was stimulated between 24-72 h of culture. Similar results were found for the rate of gene transcription using nuclear run-on elongation assays. Thus, glucocorticoid both inhibit and stimulate SP-A gene transcription in rabbit lung, and the temporal pattern of the biphasic effect is opposite to that occurring in human tissue.

The effects of in vivo glucocorticoid treatment on SP-A production have also been examined in rats by several investigators (Phelps et al., 1987a, 1991a; Floros et al., 1989; Fisher et al., 1991; Schellhase & Shannon, 1991; Shimizu et al., 1991). A 24-h exposure to dexamethasone (200 μg/kg body weight) in vivo increases the lung content of SP-A protein and mRNA in day 22 gestational age fetuses (term) and in 1-day-old

neonatal rats (Phelps et al., 1987a). Similar magnitudes (~2-fold) of the effect are observed with adult rats (Floros et al., 1989, Fisher et al., 1991). In contrast, Shimizu and colleagues showed that dexamethasone treatment increased SP-A contents when 19 and 21 day gestation fetal rats were used. Adult and neonatal 1 and 3 day rats showed no response to glucocorticoids in terms of SP-A protein synthesis. Interestingly, with neonatal 5 day old rats there was an small but significant increase in SP-A contents (Shimizu et al., 1991). Response of SP-A contents to glucocorticoids may reflect the effects of the hormone on translational and post-translational levels, since adrenalectomy of adult rats did not alter SP-A mRNA levels but decreased SP-A contents (Fisher et al., 1991). Increasing dexamethasone from 2 μg/kg to 20 mg/kg caused a dose-dependent increase in SP-A protein and mRNA contents (Floros et al., 1989, Fisher et al., 1991). The response of fetal lung to in vivo glucocorticoid treatment, however, seems to be as complex as observed with in vitro studies. Maternal dexamethasone (1 mg/kg/day) (Schellhase & Shannon, 1991) given for 1 and 3 days during the pseudoglandular stage of fetal lung development (14 and 16 days gestations, see Fig. 1.5) resulted in a precocious appearance of detectable fetal SP-A mRNA that was paralleled by an increase in fetal SP-A content. SP-A accumulation during the canalicular stage (day 19 gestation) of rat fetal lung development is dependent upon the duration of prior maternal treatment. Maternal dexamethasone (1 mg/kg/day) given for 3 and 5 days, but not 1 day, prior to sacrifice during the canalicular stage results in an increase in fetal lung SP-A protein content. This occurs without a concomitant increase in the abundance of SP-A mRNA (Schellhase & Shannon, 1991). The biphasic effect of glucocorticoids on SP-A mRNA

production is also observed with in vivo systems. An increase in the content of SP-A and translatable SP-A mRNA in gestational day-19 fetal rat lung is observed with a 24-h exposure to dexamethasone at a dose of 0.2 mg/kg (Phelps et al., 1989) but not of 1 mg/kg (Schellhase & Shannon, 1991). Inhibitory effects of glucocorticoid on SP-A production are not observed with different treatment times up to 5 days (Schellhase & Shannon, 1991, Fisher et al., 1991).

In summary, the effect of glucocorticoids on SP-A synthesis is complex, as indicated by the biphasic phenomena observed in different experimental systems. The mechanisms of the effects remain unclear except that the hormone actions appear to be mediated by its receptor and that mechanism such as mRNA stability regulation may be involved. Whether this latter mechanism occurs in every stage of lung development is not clear, since it was only elucidated in studies using early human fetal lung tissues (Boggaram & Mendelson, 1989).

(b) SP-B and SP-C

Glucocorticoids have marked dose-dependent stimulatory effects on the levels of SP-B and SP-C mRNA in human fetal lung in vitro (Liley et al., 1989; Whitsett et al., 1987c). This is in contrast to their complex effects on SP-A gene expression. At a concentration of 10^{-7} M, that causes a pronounced inhibition of the levels of SP-A mRNA, dexamethasone markedly increases SP-B and SP-C mRNA levels in 18/22 (gestation/term) days fetal rat lung explants (Floros et al., 1991; Veletza et al., 1992). The effects of dexamethasone are also time-dependent, with half-maximal stimulation

occurring at 14 h for SP-B and 19 h for SP-C (Liley et al., 1989). Dexamethasone also produces a dose- and time- dependent increase in SP-B mRNA and protein contents in vivo in rat lung (Phelps & Floros, 1989, 1991a; Connelly et al., 1991; Shannon et al., 1990; Shimizu et al., 1991), as well as in the human H441 (O'Reilly et al., 1991, 1988) and H820 cell line (O'Reilly et al., 1989b). An increase in mRNA stability in the presence of glucocorticoids has been postulated based on the result of a study using the H441 cell line (O'Reilly et al., 1991). This study demonstrated that the approximately 100-fold increase in SP-B mRNA upon dexamethasone treatment (50 nM) was accompanied by only a 4-fold increase in transcription of the SP-B gene. SP-B mRNA accumulation is dependent upon continued protein synthesis, suggesting that a relatively labile protein(s) is required for the induction and maintenance of high levels of SP-B mRNA in H441 cells following glucocorticoid treatment (O'Reilly et al., 1991).

In summary, results of studies on the effects of glucocorticoids on surfactant protein expression obtained from different systems are essentially consistent in terms of the direction of hormone responses. The magnitudes of the effects, however, differ from one system to another. The general trend is that the highest magnitude in the response to hormone treatment was found with the cell line system, followed by organ culture system. The in vivo studies demonstrated the lowest response to hormonal treatment. These may be due to the fact that the cellular environment plays a major role in modulating the program of hormone responses set during differentiation of the target cell (Johnson & Baxter et al., 1978), as well as that very low basal level of surfactant protein expression is usually observed in the cell line models. Regulation of surfactant protein

gene expression should be examined in these three different systems under different conditions in order to elucidate a complete understanding of the underlying mechanisms.

1.4.2.2. Regulation by other hormones and factors

Cyclic AMP: It has been shown that cyclic AMP analogues such as dibutyryl cyclic AMP, 8-bromo cyclic AMP and dibromo cyclic AMP at concentrations of 0.1-1 mM can markedly increase SP-A RNA and protein contents. This effect has been detected in both human (Whitsett et al., 1987a, 1987c; Ballard et al., 1990; Liley et al., 1989; Odom et al., 1987) and rabbit (Boggaram et al., 1988a, 1988b; Mendelson et al., 1986) fetal lung explants. Similar effects are also shown with agents that increase endogenous cyclic AMP levels, by activating adenylate cyclase (forskolin and terbutaline) or inhibiting phosphodiesterase (isobutylmethylxanthine) (Mendelson et al., 1986; Liley et al., 1988a; Odom et al., 1987). A marked effect on SP-A gene transcription by cAMP was also observed (Boggaram & Mendelson, 1988b). In contrast, the stimulatory effect of these agents on expression of SP-B and SP-C RNA was much smaller than for SP-A (Lilay et al., 1989; Whitsett et al., 1987c). Dexamethasone (10^{-9} M) in combination with cyclic AMP further increased the rate of transcription and the levels of SP-A RNA and protein compared to that with either agent alone (Mendelson et al., 1986; Boggaram et al., 1989; Lilay et al., 1989); higher concentrat ons of dexamethasone (10^{-6} M) reduced the cyclic AMP stimulated accumulation of SP-A RNA and protein similar to the effects of dexamethasone alone (Whitsett et al., 1987a; Boggaram & Mendelson, 1988b; Boggaram et al., 1989; Odom et al., 1988).

Epidermal growth factor (EGF): Dose-dependent increases in both SP-A mRNA and protein levels have been observed when human fetal lung explants were treated with EGF (0.1-10 ng/ml) (Whitsett et al., 1987d). The magnitude of the increase was similar to that induced by cyclic AMP. Coadministration of dexamethasone greatly reduced the stimulatory effect by EGF alone. The effects of EGF on the expression of SP-B and SP-C have not been investigated.

Interferon γ (IFN-γ) has also been shown to stimulate expression of SP-A in human fetal lung explant cultures (Ballard et al., 1990). IFN-γ increases SP-A protein and RNA in a dose-dependent manner (0.5-100 ng/ml). Low concentrations of dexamethasone (10^{-9}M) in combination with IFN-γ increased SP-A protein in a synergistic manner. The stimulatory effects of IFN-γ was specific for SP-A. No significant effects of the agent on SP-B and SP-C RNA or on phosphatidylcholine synthesis were observed.

Factors that inhibit surfactant protein expression: TGF-β deceases SP-A mRNA and protein in fetal lung explant cultures (Whitsett et al., 1987b); effects on SP-B and SP-C were not investigated. *Insulin* has also been shown to cause a dose-dependent (2.5-250 ng/ml) decrease in SP-A protein in human fetal lung explants (Snyder & Mendelson, 1987b). The effects of insulin on SP-B and SP-C are unknown. *Phorbol 12-myristate 13-acetate (TPA)* causes a dose-dependent decrease in SP-A and SP-B RNA and protein in the H441 tumor cell line (Whitsett et al., 1992). The inhibitory effect of TPA is detected within 2 h and results in a significant decrease in the half-lives of SP-A and SP-B mRNAs. The effect of TPA on SP-A and SP-B RNA stability required ongoing

transcriptional activity; but it is unclear if TPA also exerts transcriptional effects on these genes. The time course of the lowering of SP-A RNA levels by *TNF-α* is similar to that reported for TPA (Whitsett et al., 1992). TNF-α decreases SP-A protein and RNA in a dose-dependent manner (1-25 ng/ml) while increasing expression of manganese superoxide dismutase RNA. The inhibitory effects of TNF-α and TPA are consistent with involvement of a protein kinase C-dependent pathway and may ultimately be mediated through AP1 binding sites (Merriott & Brady, 1989; Curran & Franza, 1988) which have been identified in the first intron and the 5'-flanking sequences of the SP-A (White et al., 1985) and SP-B (Pilot-Matias et al., 1989) genes respectively.

CHAPTER 2 - MATERIALS AND METHODS

2.1. Materials

2.1.1. Enzymes and Chemicals

All chemicals used were of reagent grade. Radiochemicals were obtained from New England Nuclear, except for [^{35}S]methionine + [^{35}S]cysteine which were from ICN. Restriction enzymes and other modifying enzymes were purchased from Pharmacia, Boehringer-Mannheim or Bethesda Research Laboratory, as were the buffers for the restriction enzymes. Random-prime labelling reagents were purchased from Pharmacia. Reagents for polyacrylamide and agarose gel electrophoresis came from BDH Chemicals. Taq DNA polymerase for PCR, T7 RNA polymerase, nucleoside triphosphates and buffers for in vitro transcription assays and rabbit reticulocyte lysates for in vitro translation were from Promega. Rabbit antisera (#R10 and #28656) raised against bovine mature SP-B peptide (Whitsett et al., 1986) and a monoclonal antibody (#1B9) against human mature SP-B dimer forms were kind gifts from Dr. J.A. Whitsett of University of Cincinnati.

2.1.2. Plasmids, bacteriophages and bacterial strains

The plasmids and bacteriophages used in this work, pUC19, M13mp18, M13mp19 and Bluescript SK$^+$, were obtained commercially. A pUC19 plasmid containing a rabbit SP-B insert was prepared by Ms. C. Richardson and Ms. C. Ford of this laboratory (Xu et al., 1989). A second pUC19 plasmid containing rabbit 18s rDNA

was obtained by reverse-PCR (section 2.10). A pBR322 vector inserted with mouse β-actin cDNA was a kind gift from Dr. D.T. Denhardt (Rutgers' University, New Jersey). The insert was later transferred into Bluescript.SK$^+$ at the Pst I site for use. Bluescript.SK$^+$ vector containing rabbit SP-A, SP-B or SP-C inserts in both orientations were obtained from Mr. I.H. Connelly of this laboratory (Connelly et al., 1992). The Bluescript.SK$^+$ clones containing the rabbit SP-B inserts were used for making sense/antisense cRNAs. M13mp18 and M13mp19 were used as sequencing vectors. Plasmid containing inserts were selected for with medium containing 50 μg/ml ampicillin (amp^R). The Bluescript.SK$^+$, M13mp18 or M13mp19 clones containing inserts were selected for in medium containing isopropyl-β-D-thiogalactopyranoside (IPTG) (0.26 mM) plus 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (0.03%).

Escherichia coli strain JM103 was used as host for M13 phages. It has the genotype *Δ(lac-pro) thi strA supE endA1 sbcB15 hsdR [F'traD36, proAB, lacI^{q}ZΔM15]* (Hanahan 1983). The E. coli strain JM109 was used as host for pUC19 and Bluescript.SK$^+$. The genotype of JM109 is *recA1 endA1 syrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB) (r_k^-, m_k^+) lambda$^-$ [F'traD36 proAB$^+$ lacI^{q}ZΔM15]* (Messing et al., 1985).

JM103 was grown in yeast-tryptone (YT) broth (0.8% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl). JM109 was grown in Luria broth (LB). Cultures were grown at 37°C with aeration. Strains were stored in medium containing 15% glycerol at -70°C. M13 phage stocks were prepared by incubation of an agar plug containing the M13 plaque with JM103 in LB overnight at 37°C. Cells and agar were removed by

centrifugation (10,000 x g for 5 min) and the supernatant stored at 4°C.

2.1.3. Animals

New Zealand White rabbits of known duration of pregnancy (day of breeding being designated day 0) were purchased from Rieman's Fur Ranch (St. Agatha, ON. Canada) and were maintained in the animal care facility for at least one day prior to killing. The doe was sacrificed with sodium pentobarbital (500 mg) intravenously. The fetus was decapitated following uterotomy and the lungs were removed and placed in cold 1x PBS (phosphate buffered saline). Airways and major blood vessels were dissected free and discarded. Lung tissue from fetal rabbits of 26 and 30 days gestational ages were processed under sterile conditions for organ culture studies (see Sect. 2.2.). Lung tissue from fetuses of 22, 24, 26, 28 and 30 days gestational ages and 1 day old neonates as well as adult (2.5 kg) rabbits were collected to study developmental changes in SP-B gene transcription and mRNA levels (see Sections 2.3.2. and 2.9.1.).

2.2. Organ culture

The method employed for organ culture was a combination of those described previously by Whitsett et al. (1987c) and Snyder et al. (1981). The whole process was conducted under sterile condition and lung tissue was kept on ice before incubation was initiated. Briefly, lung tissues from fetal rabbits of 26 or 30 day gestational ages were trimmed, rinsed in 1x PBS, pooled and chopped into pieces of approximately 1 mm^3 using a McIlwain tissue chopper. Approximately 0.5-0.7 g tissue was distributed evenly

on nylon membranes supported by stainless-steel grids in a 6-well tissue culture plate (Gibco, Grand Island, N.Y.) and rinsed twice with Hank's balanced salt solution. The tissue was cultured in Waymouth's MB752/1 (Gibco) at 37°C in an atmosphere of 5% CO_2 and 95% air. Routinely, the tissue was first allowed to settle at 37°C for ~ 1 h then placed on a rocker platform (Bellco) and was rocked at 3 cycles per minute for the rest of the incubation period. The medium was changed daily. For studies using actinomycin D to measure SP-B mRNA turnover, the inhibitor was added to the culture when it was initiated. In studies on the effects of glucocorticoids on surfactant protein production, dexamethasone (10^{-8}M or 10^{-6}-10^{-10} M) and/or cycloheximide (10 μM) or actinomycin D (5 μM) were added from day 1 onwards. Lung explants were harvested at various times for measurement of surfactant protein mRNA levels and their gene transcription rates. The DNA and protein contents of the explant tissue were determined using standard procedures (Lowry et al., 1951; Karsten & Wallenberger, 1977).

2.3. Nucleic Acid Isolation

2.3.1. Isolation of plasmid and phage DNA

E. coli host cells containing plasmid DNA were grown overnight in medium containing the antibiotic ampicillin at 50 μg/ml. Small scale plasmid preparations were obtained by the method of Maniatis et al. (1982). Plasmid DNA was released from bacterial cells by lysozyme digestion (4 mg/ml of lysozyme, 50 mM glucose, 10 mM EDTA, pH 8.0, 25 mM Tris-HCl, pH 8.0) followed by base (NaOH/SDS)/acid (KoAc) treatment to remove cellular proteins. Plasmid DNA was then purified by

phenol/chloroform extraction and ethanol precipitation. For large scale plasmid preparations, cleared lysate of chloramphenicol-amplified cells containing supercoiled DNA was prepared by standard methods (Maniatis et al., 1982). Cellular RNA was removed by RNase A digestion. Non-nicked circular plasmid DNA was separated from protein, RNA and cellular DNA in a three-step protocol consisting of exonuclease III digestion, treatment with BND (benzoylated naphthoylated DEAE)-cellulose (Sigma) in 1 M NaCl and then phenol extraction followed by alcohol precipitation of the remaining supercoiled DNA (Kamper et al., 1985). The purity of the isolated plasmid DNA was examined by gel electrophoresis.

M13 RF (replication form) DNA was prepared using the same procedure as for small scale plasmid DNA preparation after infection of JM103 with the phage stock and growth in YT medium overnight at 37°C. Single stranded M13 phage DNA was prepared as follows. 1.5 ml of JM103 cells were infected with M13 phage for 5-6 h at 37°C and the cells removed by centrifugation (10,000 x g for 5 min). The phage (1.2 ml) were precipitated by addition of 0.3 ml of 25% polyethylene glycol (PEG) 6000, 2.5 M NaCl, incubated at room temperature for 15 min then 4°C for 5 min, and centrifuged at 10,000 x g for 10 min. The pellet was resuspended in 0.48 ml of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) containing 0.5 % sarkosyl, reprecipitated with 0.12 ml of PEG solution as above, and resuspended in 0.1 ml of TE. After extraction with phenol and phenol/chloroform, the DNA was precipitated with ethanol (Yanisch-Perron et al., 1985).

All DNAs were kept in H_2O at -20°C for storage. The concentration of DNA was

determined spectrophotometrically at 260 nm.

2.3.2. Isolation of RNA from tissue

Total RNA was isolated from lung or liver tissue using the CsCl sedimentation method (Chirgwin et al., 1979) or the single-step Guandinium-Acid-Phenol-Chloroform (GAPC) method (Chomczynski & Sacchi, 1987). Briefly, the tissue was homogenized using a Polytron in 4 volumes of guanidinium solution prior to centrifugation through 5.7 M CsCl cushion or GAPC extraction. The RNA pellet recovered after CsCl sedimentation was routinely extracted using phenol, phenol-chloroform, and chloroform (24:1 with isoamylalcohol). RNA precipitated with ethanol was then dissolved in DEPC-treated H_2O and stored at -70°C. The concentration of RNA was determined spectrophotometrically at 260 nm.

In some cases, cytoplasmic RNA was isolated as follows: the tissue was homogenized in Tris-sucrose buffer (see Section 2.9.1.) using a hand-driven Dounce Homogenizer. The supernatant (cytoplasm) was collected after centrifugation at 600 x g for 5 min to pellet the nuclei. Cytoplasmic RNA was then precipitated with 0.1 M NaCl and 2 volumes of ethanol at -20°C overnight (Boggaram et al., 1988b). After centrifugation at 2000x g for 15 min at 4°C, the pellet was dissolved in 4 volumes of the guanidinium solution and subsequently subjected to CsCl centrifugation or acid-phenol extraction.

2.4. DNA restriction, Modification, Recombination and Transformation

Restriction endonucleases were used according to suppliers' instructions (using 3-4 U/μg DNA for 3 to 4 h incubation). After incubation, an aliquot was examined using agarose gel electrophoresis to ensure complete digestion.

Vectors for subcloning were routinely dephosphorylated at the 5'end with calf intestinal phosphatase (CIP) according to Maniatis et. al. (1982).

Ligations of DNA fragments were performed using T4 DNA ligase at room temperature for 2 h for fragments with sticky ends or overnight at 14°C for fragments with blunt ends in a 10 μl reaction in 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP. Typically, 8 units of T4 DNA ligase were used for total of 40 ng vector DNA and a 3- to 5-fold molar excess of gel-purified insert DNA.

Ligated plasmid DNA was introduced into *E. coli* by the calcium chloride procedure (Maniatis et al., 1982). Freshly prepared competent cells were normally used. Transformed cells were selected by growth on agar plates containing the appropriate antibiotic to which resistance was conferred by the introduced plasmid. M13 DNA was introduced into JM103 as follows: JM103 was made competent and mixed with DNA for 30 min at 4°C. After the 2 min of heat shock at 42°C, 200 μl of transformation mixture was then plated in 2.5 ml of molten YT top agar (0.7 %) containing about 10^7 JM103 cells, 0.5 mM IPTG, and 0.03% X-gal. In the presence of the inducer IPTG and the substrate X-gal, expression of β-galactosidase from the *lacZ* gene in non-recombinant M13 produces blue plaques, while recombinant phage were identified as colourless plaques due to disruption of the *lacZ* gene.

Selected recombinant plasmids and phage were identified by restriction mapping

or Southern analysis (Section 2.6.2). The relative orientations of inserts in M13 phages were determined by complementary tests as described by Messing (1983).

2.5. Gel Electrophoresis of Nucleic Acids and Proteins

2.5.1. Gel electrophoresis of DNA

Agarose gel electrophoresis: DNA with or without restriction enzyme digestion was loaded onto 0.8 to 1.2% horizontal agarose gels prepared in 1x TBE buffer (89 mM Tris-borate, pH 8.0, 2 mM EDTA, pH 8.0) in the presence of DNA gel loading buffer (7% sucrose, 0.03% bromophenol blue and 0.03% xylene cynanol). The gel was run in 1x TBE under constant voltage at 100 V for 3-4 h or 25 V overnight. DNA was then visualized by staining the gel with ethidium bromide (0.5 μg/ml) and viewed and photographed under short wavelength ultraviolet light (302 nm).

For recovery of DNA fragments from agarose gels after viewing, a slice of the gel was removed so that a well was formed directly below the desired DNA band. A semi-permeable dialysis membrane (Spectra/Por membrane, Spectrum Medical Industries, Inc.) was slid into the well. The DNA fragment was run into the well, which contained small amounts of running buffer, at 100 V for ~30 min. The buffer in the well was collected and extracted twice with isobutanol to remove ethidium bromide, then with phenol, phenol/chloroform, and chloroform. DNA was recovered by ethanol precipitation. In some cases, low-melting point agarose gel electrophoresis was used to isolate DNA fragments (Maniatis et al., 1982).

Polyacrylamide gel electrophoresis: Small DNA fragments (< 1000 bp) were

sometimes analyzed using 6% polyacrylamide gels run in 1x TBE buffer at 200 V for 20 to 40 min. In some cases, DNA fragments were recovered from polyacrylamide gels according to the method of Maniatis et al. (1982).

Sequencing gels are described in Section 2.8. "DNA Sequencing and Sequence Analysis".

2.5.2. Gel electrophoresis of RNA

RNA was treated with 1.0 M glyoxal-solution (Thomas, 1983; McMaster and Carmichael, 1977) prior to gel electrophoresis. Two μg of glyoxalated RNA were separated on a 1.1% or 1.5% agarose gels in circulating sodium phosphate buffer (10 mM, pH 6.4) and viewed after ethidium bromide staining following alkaline fixation of the gel. Normally a duplicate gel with ten μg of RNA per sample was also transferred to Nytran membranes (Schleicher & Schuell) (see Section 2.6.2).

To analyze radiolabelled cRNA made by in vitro transcription, the cRNA was run on a 6% polyacrylamide vertical gel containing 7 M urea. The gel was then soaked in 10x gel volumes of 1 M sodium salicylate, pH 5-7 and dried under vacuum before being subjected to autoradiography.

2.5.3. Gel electrophoresis of proteins

^{35}S-labelled protein samples either before or after immunoprecipitation (Section 2.12.1) were analyzed on an 11% SDS-polyacrylamide gel (Laemmli, 1970) containing 7 M urea. After electrophoresis, the gel was fixed in acetic acid:methanol:water (2:9:9)

for 1 h. Fluorography was performed using PPO in DMSO (22.2%, w/v) for 3 h after the gel was presoaked in DMSO twice for 30 min. The gel was then dried under vacuum and subjected to autoradiography.

For Western blot analysis, lung tissue was homogenized in 10x volume of 1x PBS containing 1 mM PMSF (Shimizu et al., 1991). Samples with appropriate amounts of protein (determined by the assay of Lowry et al., 1951) were loaded onto a 15% Laemmli gel. For viewing, one of the duplicate gels was stained with Coommassi Blue R-250 for 30 min at room temperature and destained overnight at room temperature followed by photography. The protein samples on the other identical gel were electro-transblotted (BioRad) at 70 V for 3 h onto a nitrocellulose membrane (Schleicher & Schuell) in gel running buffer containing 20% methanol. The blot was then subjected to immunodetection (see Section 2.12.2). In some cases, in vitro translation samples containing approximately 25,000 dpm were analyzed using the above method.

Molecular weight markers (BioRad & New England Nuclear) used were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), lactoglobulin A (20 kDa) and lysozyme (14 kDa). Sizes on figure 5.8 are indicated in kilodaltons.

2.6. Nucleic Acid Hybridization

2.6.1. Preparation of radiolabelled DNA probes

Random-primed labelling: The ^{32}P-labelled DNA probes for rabbit SP-A, SP-B, SP-C, 18s rDNA (see section 2.10) or for mouse beta-actin (a kind gift from Dr. D.T.

Denhardt, Rutger's University) were made by the oligolabelling reaction (Feinberg and Vogelstein, 1984) using Oligo-labelling kits from Pharmacia. For the reaction, 50 ng of DNA fragment purified from agarose or polyacrylamide gel were heat denatured and incubated at 37°C for 2.5 h or overnight, in 50 μl mixture of 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.2 M Hepes, 5.4 OD units of hexadeoxyribonucleotides, 20 μM each of dATP, dGTP, and dTTP, 20 μg of BSA, 5 μCi [α-^{32}P]dCTP (3000 Ci/mmol, DuPont-NEN), and 2 units of Klenow fragment of *E. coli* DNA polymerase I. The reaction was stopped by addition of 5 μl of 0.2 M EDTA, pH 8.0) and dilution with H_2O. The labelled DNA was separated from unincorporated ^{32}P-dCTP by column chromatography using Sephadex G-50 column (Pharmacia) with H_2O (Maniatis et al., 1982). The specific activity of the probes, determined by TCA precipitation, was found to be $\sim 10^8$ cpm/μg DNA.

End-labelling: DNA markers were sometimes labelled at the 3' end with [α-^{35}S]dATP according to Maniatis et al. (1982). The reaction was carried out at room temperature for 10 min in the presence of 50 mM Tris-HCl, pH 7.0, 10 mM $MgSO_4$, 0.1 mM DTT, 4 μl of [α-^{35}S]dATP (1350 Ci/mmol, DuPont NEN), 5 U of Klenow fragment of DNA polymerase I (Pharmacia) and 0.5 μg of the DNA fragments. The labeled DNA was purified by extraction with phenol/chloroform and ethanol-NaAc (sodium acetate) precipitation.

2.6.2. Southern, Northern transfer and slot blotting

After electrophoresis, DNA or RNA samples fractionated on the agarose gel were

transferred to a Nytran membrane using the classical Southern method (Maniatis et al., 1982) or a VacuGene apparatus (Pharmacia LKB) in 20x SSC (1x SSC = 50 mM NaCl, 90 mM NaH_2PO_4 and 1 mM EDTA). Once the blot was air-dried, it was baked at 80°C for 0.5 h. The DNA blot was then incubated with 50 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, pH 8.0 and 0.1% SDS (sodium dodecyl sulfate). For Northern transfer blots, the membrane was wetted with H_2O and UV-cross-linked under 302 nm ultraviolet light for 3 min. The blots were then washed in 20 mM boiling Tris-HCl, pH 8.0 to remove the glyoxal residues (Thomas, 1983) and cooled to room temperature.

To quantify RNA samples, slot blots were prepared in duplicate slots with 5 μg of total RNA (see Fig. 4.3) in each slot from a single dilution of glyoxalated RNA and applied to nitrocellulose filters in 10x SSC by the method of Maniatis et al. (1982). The blot was baked 2 h at 80°C and then washed with boiling Tris-HCl, pH 8.0.

DNA slot blots, used in the nuclear run-on assays, were prepared as follows: appropriate amounts of linearized DNA were heat denatured in the presence of 50 mM NaOH. The DNA was then neutralized with ammonium acetate before being applied to nitrocellulose filter. The blot was baked 2 h at 80°C before use.

Blots were stripped of the previous probe before being hybridized with another probe. The stripping procedure was to shake the blot in (1) boiling Tris-HCl, pH 8.0 (20 mM) and cooling to room temperature, twice; then (2) 0.1x SSC and 0.5% SDS at 65 C for 1-2 h.

2.6.3. Hybridization and Autoradiography

DNA blots were pre-hybridized at 42°C for over 4 h in 50% deionized formamide, 5x SSC, 5 X Denhardt's solution (1x = 0.1 g Ficoll, 0.1 g polyvinylpyrrolidone, 0.1 g BSA) and 10 mg/ml salmon sperm DNA. RNA blots were pre-hybridized at 42°C overnight in 50% deionized formamide, 5x SSC, 50 mM sodium phosphate, pH 7.0, 5x Denhardt's solution, 1 % SDS and 10 μg/ml poly U (Pharmacia). Hybridization was conducted in the same respective solution with the ^{32}P-labelled DNA probe (1-5 X 10^5 cpm/ml) for 18 h at 42°C.

After hybridization, filters were washed two times (15 min each) in a large volume of 2x SSC and 0.1 % SDS at room temperature, once in 1x SSC and 0.1 % SDS at 42°C, once in 0.1x SSC and 0.1 % SDS at 65°C and exposed for autoradiography with an intensifying screen at -70°C for appropriate periods of time.

Autoradiograms of slot blots were scanned by laser densitometry, using a Ultrascan densitometer (Pharmacia). Only densitometry values falling within the linear range of film exposure were included in the analysis. Hybridization values for SP-A, SP-B or SP-C were normalized to 18S rRNA or β-actin hybridization values.

2.7. Solution Hybridization Assay

2.7.1. In vitro transcription

The rabbit SP-B cDNA insert was cloned into the EcoRI site of Bluescript.SK$^+$ plasmid in both orientations. The plasmid was then linearized with XbaI restriction enzyme. The linear form of the clone was purified by phenol extraction and the DNA recovered by ethanol precipitation. Transcription reactions for sense or anti-sense SP-B

cRNAs were conducted with T7 RNA polymerase. One μg of the DNA template was added to a final volume of 20 μl of T7 RNA polymerase in vitro transcription cocktail as specified by the manufacturer (Promega/Fisher Scientific Ltd.). The cRNA generated was extracted with phenol and precipitated with ethanol. An aliquot of the product was analyzed on a 6% polyacrylamide gel containing 7 M urea to confirm the RNA was of the desired length (Fig. 3.5). Radioactive cRNA probes were produced by substituting [^{35}S]UTP for the cold nucleotide. Two hundred and fifty microcuries of [^{35}S]UTP (1320 Ci/mmol) were concentrated to approx. 10 μl using a cooled Savant speed-vac jet before addition to the reaction. The product was precipitated with ammoniumacetate-ethanol. The integrity of the probe was confirmed by autoradiography following gel electrophoresis under the above conditions (see Fig.3.5).

2.7.2. Solution hybridization assay

Solution hybridization assays were carried out according to Connelly et al. (1991) and Durnam and Palmiter (1983) to measure SP-B mRNA levels in experiments shown in Section 4.2.5. Total RNA isolated from lung explants from the SP-B mRNA turnover experiments was used. For each sample, 10 μg of total RNA were assayed in triplicate. Samples for the standard curve contained 10 μg yeast RNA and serially diluted synthetic SP-B cRNA standards. Ten micrograms of adult liver RNA were also assayed to provide a measure of background. The RNA to be assayed was incubated in 100 μl of 0.6 M NaCl, 20 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2% SDS and labelled antisense SP-B cRNA (75,000-100,000 cpm) at 70°C for 16-18 h, then transferred to 37°C for 30 min.

One ml of 0.3 M NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 75 μg/ml denatured salmon sperm DNA, 25 μg/ml RNase A and 20 U/ml RNase T1 was added, and the tubes were incubated at 37°C for 1 h to digest free probe. Hybrids were precipitated by addition of 370 μl of 30% cold TCA, and chilled at 4°C for at least 30 min. Precipitated nucleic acids were recovered by centrifugation (30 min at 10,000 x g and 4°C), washed with 1 ml of cold 5% TCA/0.01% Triton X-100, and resuspended in a bath sonicator with 100 μl of 20 mM NaOH. This solution was counted in 4 ml Beckman (Missassaga, Ontario, Canada) Ready Safe Scintillation fluid. Approximately 50-60% of the total labelled cRNAs could be precipitated after hybridization with the standard RNA used to construct the standard curve. Background radioactivity, observed with yeast total RNA or rabbit liver RNA, was between 5% and 10% of the total added radioactivity. The standard curves were linear between approximately 1 and 100 ng of SP-B cRNA, corresponding to the amounts of SP-B mRNA in 10 μg of total RNA (when SP-B mRNA represents 0.1% to 0.01% of total messenger). When the same samples were examined in different assays, the values agreed within 15%.

2.8. DNA sequencing and sequence analysis

The DNA sequence of rabbit SP-B clone MR6M in the single stranded M13 vector was obtained by the dideoxynucleotide chain termination method of Sanger et al. (1977) using [α-^{35}S]dATP and a modified bacteriophage T7 DNA polymerase from a Sequenase kit (United States Biochemical). A universal 17-mer oligonucleotide complementary to the M13 polylinker was used as the primer. For some portions of the

sequence, synthetic oligonucleotides were designed (synthesized by Dr. G. Mackie, Biochemistry, University of Western Ontario) and served as primers for the sequencing reaction.

Products of the sequencing reactions were analyzed on 8% polyacrylamide gels containing 7 M urea, in a buffer composed of 50 mM Tris-HCl, pH 8.3, 1 mM EDTA. Wedge gels (0.2-0.4 mm thick) were used. Gels were fixed in 10% acetic acid, 10% methanol, dried onto Whatman 3mm paper, and exposed to XAR-2 film. Sequences were read by eye with at least two proofreadings. The PC Gene Program (IntelliGenetics Inc.) was used for sequence analysis.

2.9. Nuclear Run-on Assay

2.9.1. Nuclei isolation

Nuclei were isolated according to the method of Boggaram and Mendelson (1988b) from fresh adult liver, lung explant tissues of each treatment group, or minced (1 mm^3) fresh lung tissue removed from fetuses of different gestational ages, neonatal and adult rabbits. All procedures were performed on ice at 4°C. The tissues were homogenized in 10 volumes/gram tissue of homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 0.1% (v/v) Triton X-100) using a glass/glass Dounce homogenizer and 10 hand-driven strokes. The homogenate was filtered through a Pasteur pipette plugged with sterile glass wool and a crude nuclear pellet was isolated by centrifugation at 600 x g for 5 min. The supernatant was saved for isolation of cytoplasmic RNA as described in Section 2.3.2.

The crude nuclei were washed twice in homogenization buffer and finally centrifuged through 1.3 M sucrose in the homogenization buffer at 10,000 x g for 10 min (1 volume of nuclei to 3 volumes of 1.3 M sucrose buffer). The nuclei were suspended in 50 mM Tris-HCl, pH 8.0, containing 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, and 2 mM dithiothreitol. Typically, nuclei from 1 g of tissue were suspended in 1.0 ml of glycerol-containing buffer, immediately frozen, and stored at -70°C for future use. Nuclei were quantified using a haemocytometer after dilution with suspension buffer.

2.9.2. Transcriptional run-on assay

Nuclear run-on assays were conducted essentially as described by Boggaram and Mendelson (1988b) and Marzluff and Huang (1983). Nuclear run-on assays were performed in a total volume of 200 μl that contained 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 20% glycerol, 0.05 mM EDTA, 2 mM dithiothreitol, 0.5 mM each of ATP, CTP, UTP and 0.5 μM of [α-^{32}P]GTP (800 Ci/mmol) (Dupont NEN), and 2x 10^7 nuclei. Sufficient nuclei were obtained to conduct two to three reactions for each experiment. The reaction was allowed to proceed for 40 min at 30°C. The reaction was terminated by digestion with 20 mg/ml of RQ1 DNase I (Promega) for 10 min at 37°C followed by digestion with Proteinase K (100 μg/ml) in the presence of 10 mM EDTA, pH 8.0 and 0.5% SDS for 30 min at 37°C. Samples were extracted twice with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1), and the RNA was co-precipitated with 30 μg of yeast tRNA with ammonium acetate (1.25 M) and 2 volumes of ethanol. The RNA pellets were dissolved in 100 μl of 10 mM Tris-HCl, pH 7.5 and 1 mM

EDTA, pH 8.0. The labelled RNA molecules were separated from unincorporated nucleotide by passage through a pre-packed G-50 column (Pharmacia) and the radioactivity was determined by scintillation counting. Equivalent amounts (typically around 1x 10^6 cpm/ml) of radioactive RNA from each treatment group or gestational age sample were taken up in hybridization buffer (40% formamide, 5x SSC, 1x Denhardt's solution, 0.1% SDS and 100 μg/ml of denatured salmon sperm DNA) and added to Petri dishes (D=35 mm) containing nitrocellulose filters which were previously slot-blotted with equal amounts (2.6 pmoles) of linearized cDNAs for rabbit SP-A, SP-B, SP-C and mouse β-actin or rabbit 18s DNA (see section 2.6.2). The filters were prehybridized overnight and then hybridized with the ^{32}P-labelled RNA transcripts at 42°C for 65 h in a shaking water bath. After routine washing (see Section 2.6.3.), the filters were washed at 37 C in 2x SSC and 0.1% SDS containing 10 μg/ml RNase A, 10 U/ml RNase T1 and then treated with Proteinase K (100 μg/ml). The filters were finally washed in 2x SSC, 0.1% SDS for 20 min at 45°C before being subjected to autoradiography. Using these hybridization and washing conditions, the intensity of the signals produced by nonspecific DNA (pUC19) was less than 5% of the experimental values (Fig. 4.4).

2.10. Reverse PCR

Reverse PCR was carried out according to (Ausable et al., 1990). Two micrograms of total RNA isolated from lung tissue of 30 days gestation were coprecipitated with 3 pmoles of downstream primer, which was specific for the 18s rDNA sequence, in ethanol overnight. The RNA and the primer were recovered by

centrifugation and resuspended in 20 μl of 80 mM Tris-HCl, pH 8.0 and 80 mM KCl and allowed to denature at 90°C 1 min then annealed by cooling slowly to 67°C, followed by incubation at 52°C for 3 h. Reverse transcription reaction was carried out in buffer (50 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 40 mM KCl, 10 mM dithiothreitol, 100 μg/ml BSA) containing 0.5 mM of each trinucleotide phosphate and 20 U of MMLV (Moloney Murine Leukaemia Virus) reverse transcriptase (Boehringer-Mannheim) at 37°C for 1 h. The reaction was stopped with 450 μl of 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 8.0. An aliquot of the product was used for the PCR reaction. The first strand DNA was incubated in 50 μl PCR reaction buffer containing 50 pmoles of each primer, 20 mM of each dNTP, 0.2 mM free Mg^{2+}, and 2.5 U of Taq polymerase (Promega). The thermal cycle was set for denaturation at 94°C for 5 min, annealing at 65°C for 2 min and polymerization at 72°C for 3 min and repeated 40 times. The PCR product was analyzed on a 6% polyacrylamide gel (Section 2.5.1) and ethidium bromide staining. The fragment was eluted from the gel (Section 2.5.1) and used directly as template for synthesis of DNA probe by the oligo-labelling assay. It was also subcloned into pUC19 for amplification after restriction with an appropriate endonuclease.

2.11. In Vitro Translation

In vitro translation took place in a reaction volume of 10 μl containing 25 μCi of [^{35}S]methionine+[^{35}S]cysteine (1000 Ci/mmol, ICN), and a nuclease-treated rabbit reticulocyte lysate according to the supplier's protocol (Promega). Between 0.5 and 1.5 μg of in vitro transcribed sense SP-B cRNA, 1.5 μg of antisense SP-B cRNA or 1 μg of

total RNA isolated from 30 day gestation rabbit lung were used in the assays. To determine the [^{35}S]methionine +[^{35}S]cysteine incorporation, a 1.0 μl aliquot of the in vitro translation reaction was diluted into 0.5 ml water and precipitated with 5 ml of 10% TCA and boiled for 10-15 min. The precipitates were collected on Whatman GF/C filters by vacuum filtration, washed with water, 5% TCA, ethanol and acetone, then dried and analyzed by standard scintillation counting. Equal counts of the translation products from each reaction were analyzed on 11% SDS-polyacrylamide gels containing 6 M urea (Section 2.5.3)

2.12. Immunodetection

2.12.1. Immunoprecipitation

The ^{35}S-labelled in vitro translation products of rabbit SP-B cRNA were immunoprecipitated as described by Weaver et al. (1988). The proteins were diluted with 10 volumes of 20 mM sodium phosphate, pH 7.0, 200 mM NaCl, 2% Triton X-100, 1% sodium deoxycholate, 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 0.75 mg/ml BSA and incubated for 60 min on ice with 5 μl of 1:512 antiserum R#10. The resulting immune complexes were collected with 11 μl of *S-aureus* protein A by incubation for 40 min on ice, followed by washing five times with 10 mM sodium phosphate, pH 7.0, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.05 M PMSF and 0.1 % SDS. The proteins were finally resuspended in sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mecaptoethanol, 0.05 mM PMSF, 5% glycerol, 0.01 % bromophenol blue). The immunoprecipitation reactions together with the

translation reactions were analyzed on a urea-polyacrylamide gel (Section 2.5.3).

2.12.2. Immunoblot analysis

Tissue homogenates (Section 2.5.3) were stored at 4°C overnight. The supernatants from low speed centrifugation, with equal amounts of total protein as determined by Lowry's assay (Lowry et al., 1951) for different samples, were loaded onto nitrocellulose membranes using a Dot Blot manifold (BioRad) according to manufacturer's instruction. The membranes or the Western blot filters obtained in Section 2.5.3. were routinely blocked for 30 min at room temperature with gelatin (3% in TBS buffer: 20 mM Tris-HCl, pH 7.5 and 0.5 M NaCl). The blots were rinsed briefly then incubated overnight at room temperature with appropriate primary antibodies in TBS buffer containing 1% gelatin and 0.05% Tween 20. Excess antibody was washed off. The blots were reacted with alkaline phosphatase-conjugated secondary antibody (BioRad) for 2 h at room temperature. Colour development was carried out in the presence of NBT (nitroblue tetrazolium) and BICP (bromo-chloro-indolyl-phosphate).

2.13. Measurement of RNA Synthesis with ^{3}H-Uridine

To evaluate the effect of various concentrations of actinomycin D on the biosynthesis of RNA in the lung tissue explants, the incorporation of radiolabelled uridine into nucleic acid was measured. Lung tissue explants from adult rabbits were incubated in Waymouth's medium at 37°C for 24 h with addition of 0, 0.75, 1.75, 2.75, 3.75 and

5 μM of actinomycin D. Approximately 0.6 μCi/ml [5,6-^{3}H] uridine (37.5 Ci/mmol; DuPont NEN) were added to the culture for the last 4 h. The tissues from each dose were then homogenized in 1x PBS to yield 10% homogenates. An aliquot was taken for trichloroacetic acid (TCA) precipitation and subsequently measurement of incorporated radioactivity.

2.14. Measurement of Protein Synthesis with ^{3}H-Leucine

In some cases, total protein biosynthesis in lung explant tissues was measured as follows: approximately 0.1 mg explant tissue was left in the 6-well culture plate at the end of incubation period and allowed to continue incubating for 3 h at 37 C in the presence of L-[4,5-^{3}H] leucine (56 ci/mmol; DuPont NEN) at a final concentration of 5 μci/ml. The tissue was subsequently washed with Hank's solution containing cold leucine (10 mM) and homogenized in 1 ml of 1x PBS containing nonradioactive leucine (10 mM) and 1 mM PMSF, using a Polytron. Total protein biosynthesis was estimated by measurement of the radioactivity after TCA precipitation of a fraction of the homogenates. Another fraction of the homogenates was utilized to determine protein content by the Lowry assay (Lowry et al., 1951).

2.15. Light Microscopy of Lung Tissue

Morphological studies of the effects of explant culture in the presence and absence of dexamethasone were performed on lung tissues collected at the initiation of or during culture. The tissue samples were randomly picked, fixed in 10% formalin, and stored

at 4°C before further processing. The tissues were processed for routine light microscopy by the Department of Pathology, University Hospital, University of Western Ontario.

2.16. Statistical Analysis

Statistical analyses were performed on raw or log-transformed data by one or two-way analysis of variance followed by Duncan's multiple comparisons using a Hewlett-Packard Analysis of Variance Statistical Package and a HP9845B computer.

CHAPTER 3 - SEQUENCING AND CHARACTERIZATION OF A RABBIT SP-B cDNA CLONE

3.1. Introduction

Surfactant-associated protein B (SP-B) is one of the two protein components present in the surfactant organic solvent ..tract preparations, which have been used in treating Hyaline Membrane Disease or the Respiratory Distress Syndrome (RDS). In most species, SP-B is identified from lung lavage fluid as an 8-kDa monomer in the presence of reducing agents or a 18-kDa dimer or higher oligomers under non-reducing conditions after SDS/PAGE (for review see Possmayer, 1984 or section 1.1.4). SP-B is synthesized in alveolar type II epithelial cells as a larger precursor of M_r 40,000 (Jacobs et al., 1987). Through proteolytic and other post-translational processing this preproprotein yields the smaller airway forms of SP-B (for review see Weaver et al., 1992).

During the time this study was being conducted, sequences for human lung cDNAs encoding SP-B were described (Glasser et al., 1987; Jacobs et al., 1987), as well as cDNAs encoding this protein from canine lungs (Hawgood et al., 1987) and from rat lungs (Emire et al., 1989). Recently, the sequence of murine SP-B cDNA has been reported (D'Amore et al., 1991). The primary amino acid sequences of bovine and porcine SP-B were also reported by Olafson et al. (1987) and Curstedt and colleagues (1988). Because the rabbit has long been used in this laboratory to study the regulation of phospholipid synthesis during fetal lung development, it was considered important to

characterize the cDNA encoding rabbit SP-B in order to be able to apply this probe to investigate the regulation of expression of the protein during fetal lung maturation.

This chapter reports the nucleotide and deduced amino acid sequences for rabbit SP-B cDNA. Further characterization of the cDNA clone by coupled transcription-translation in vitro followed by immunoprecipitation is also presented. The results indicate that the clone possesses the entire sequence coding for the preproprotein of rabbit SP-B. It has served as a probe to study the regulation of SP-B mRNA levels during lung development as presented in the later chapters. The predicted amino acid sequence is also compared with those from other mammals and discussed in detail in order to provide some structural information on rabbit SP-B mRNA and the SP-B active peptide.

3.2. Results

3.2.1. Sequence analysis of a rabbit SP-B cDNA clone.

A rabbit lung cDNA library constructed in lambda gt11 (by C. Richardson in this laboratory) was screened with ^{32}P-labelled human SP-B cDNA (gift of Dr. J. Whitsett). Screening a total of 5 X 10^4 plaques (by C. Ford in this laboratory) yielded three positive clones. Recombinant phage DNA was digested with EcoRI and examined by Southern blot hybridization. One clone was found to contain a 1.7-kb insert which hybridized with the human cDNA. The clone was inserted into phage M13 sequencing vectors (named MR6M) and sequenced by the dideoxy DNA sequencing (Sanger et al., 1977) in both orientations. In order to better analyze sequences in the centre of the clone, a set of

Figure 3.1. Partial restriction map and sequencing strategy for the rabbit SP-B cDNA clone. The entire SP-B cDNA is depicted by the bar drawing, with the 5' and 3' untranslated regions designated by open bars and the open reading frame by a darkened area. Informative restriction sites used for generating subclones are shown (E: EcoRI; S: SmaI; P: PstI; K: KpnI). The arrows below the map indicate the directions and extent of sequencing. Open arrows indicate sequences determined using M13 universal primer; solid arrows indicate sequences determined using synthetic oligonucleotide primers.

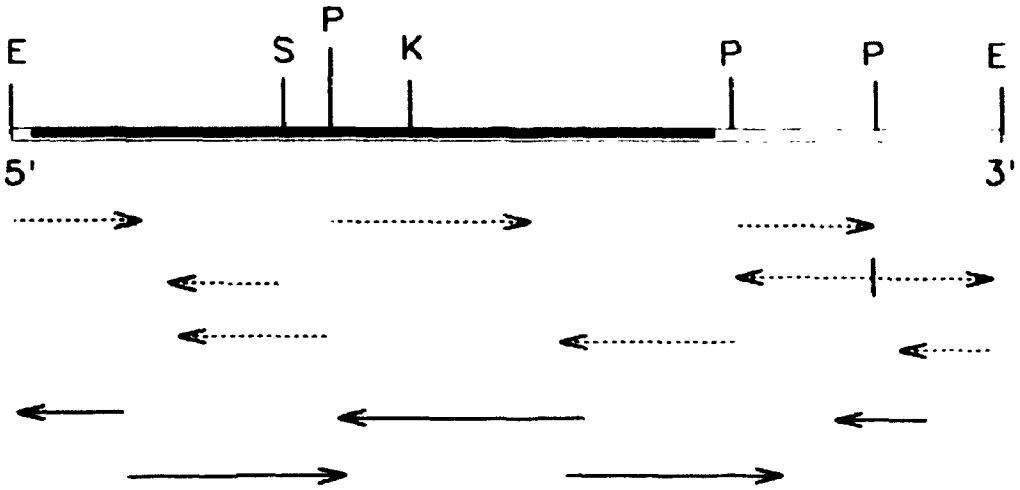
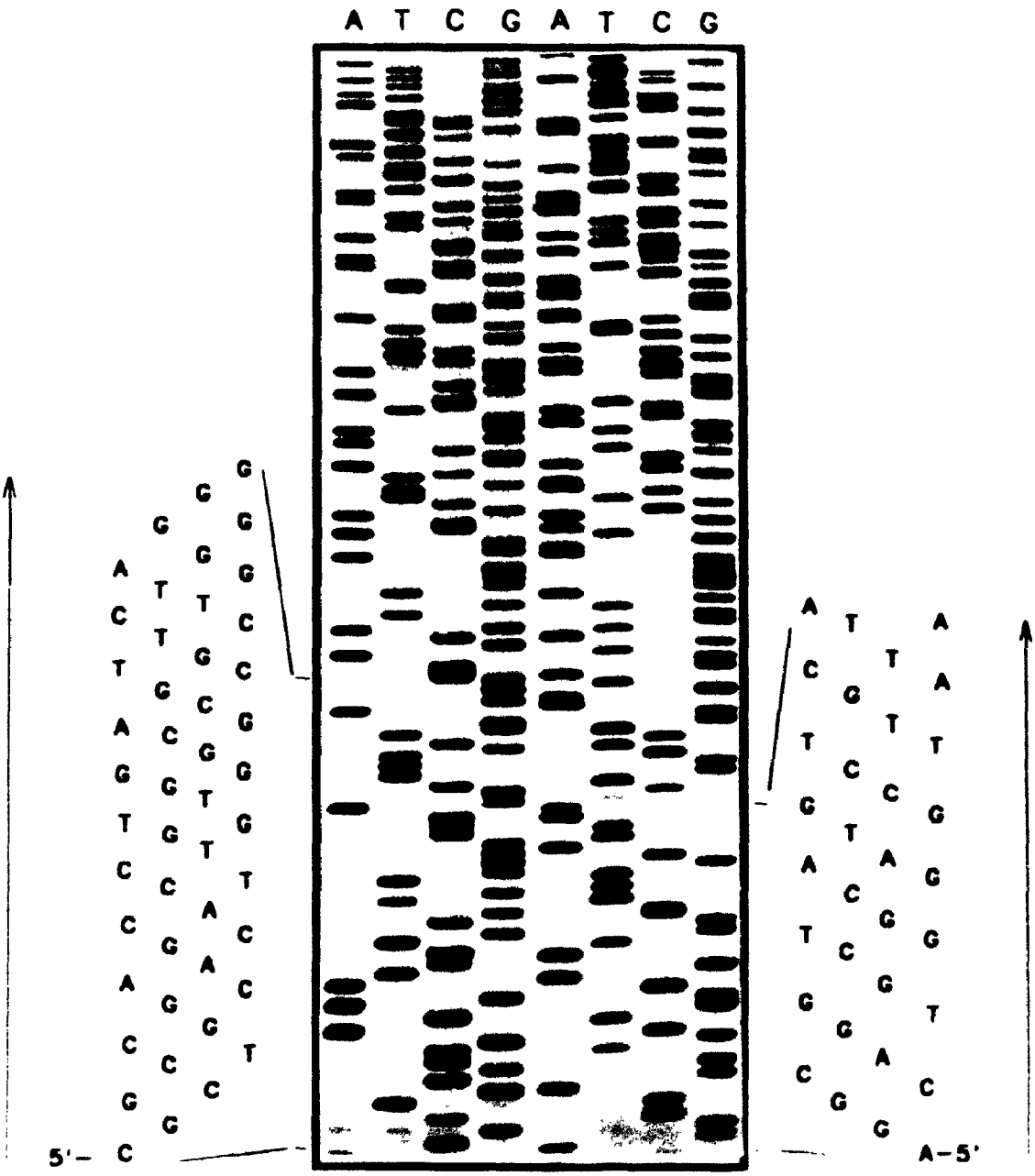

E
S
P
K
P
P
E
5'
3'

Figure 3.2. An illustrative sequencing gel for the SP-B cDNA clone. The gel shows partial sequences of two subclones of SP-B cDNA obtained using the dideoxy chain termination technique from single stranded templates produced in recombinant M13 phage. On the left side shows the inverted sequence from nucleotides 541-559 (containing sequence encoding the N-terminal cleavage site before the initiating Phe_{+1} residue of the active peptide, see Sections 3.2.1. and 3.3) and on the right side shows the inverted sequence from nucleotides 364-436 in the rabbit SP-B cDNA.


A T C G A T C G
5'–
A–5'

subclones were also obtained by digestion of the clone with the restriction enzymes shown in Fig. 3.1.. and insertion into appropriate cloning sites of the M13 vectors. Several oligonucleotides were designed according to the sequenced areas and synthesized by Dr. G.A. Mackie (Department of Biochemistry, University of Western Ontario). These synthetic oligonucleotides then served as primers to obtain sequences overlapping the subcloning sites. The partial restriction map and the sequencing strategy for the SP-B cDNA are shown in Fig. 3.1. A typical sequencing gel is shown in Fig. 3.2. The nucleotide sequence determined and the predicted amino acid sequence are shown in Fig. 3.3.

The nucleotide sequence contains a total of 1,696 nucleotides which includes a very short (2 nucleotides) 5'-untranslated region. This feature is also seen in SP-B cDNAs of other species (see Section 3.3.). A potential open reading frame which is 1,111 nucleotides in length was identified. This is followed by 577 nucleotides of the 3'-untranslated sequence. Four putative polyadenylation signals are found in this region, with three of them overlapping and in tandem. Of the two potential initiator methionines at the 5' end of the open reading frame, the first at nucleotide 3 predicts a protein of 370 amino acids and a primary translation product of 40,700 daltons including a leader-like peptide (Heijne, 1985), as determined using the method of Kyte and Doolittle (1982). The second methionine at nucleotide 251 would produce a protein of 288 amino acids with a mass of 31,680 daltons (Fig. 3.5.B). Rabbit SP-B is unusual in that it contains 37 proline and 23 cysteine residues within the 370 amino acid proprotein. The deduced active fragment for rabbit SP-B consists of 79 amino acids with a predicted molecular

Figure 3.3. Nucleotide and deduced amino acid sequences of rabbit SP-B cDNA. Nucleotides are numbered 5' to 3'. Amino acid are numbered 1-370 with the first codon for methionine at nucleotides 3-5. Sequence for the 79-amino acid active peptide is in bold type. The putative glycosylation site (nucleotides 906-914) is shadowed. The 3' untranslated region is italicized with the putative polyadenylation signals in boldface.

```
      CC ATG GCC AAG TCA CAC CTG CCG CCG TGG CTG CTG CTG CTG  47
         Met Ala Lys Ser His Leu Pro Pro Trp Leu Leu Leu Leu  13

CTG CTG CCC ACA CTC TGT GGC CCA GGC ACT GCT GTC TGG GCC ACT  92
Leu Leu Pro Thr Leu Cys Gly Pro Gly Thr Ala Val Trp Ala Thr  28

TCA CCC TTG GCC TGT GCT CAG GGC CCT GAG TTT TGG TGC CAA AGC
Ser Pro Leu Ala Cys Ala Gln Gly Pro Glu Phe Trp Cys Gln Ser

CTG GAG CAA GCA TTG CAG TGC AAA GCC CTG GGA CAC TGT CTA CAG  182
Leu Glu Gln Ala Leu Gln Cys Lys Ala Leu Gly His Cys Leu Gln  58

GAA GTC TGG GGA CAC GTG GGA GCC GAT GAC CTG TGC CAG GAG TGT
Glu Val Trp Gly His Val Gly Ala Asp Asp Leu Cys Gln Glu Cys

CAG GAC ATC GTC AAC ATC CTA ACC AAG ATG ACC AAG GAG GCC ATT  272
Gln Asp Ile Val Asn Ile Leu Thr Lys Met Thr Lys Glu Ala Ile  88

TTC CAG GAC ACC ATA CGG AAG TTT CTG GAG CAT GAG TGC GAC GTT
Phe Gln Asp Thr Ile Arg Lys Phe Leu Glu His Glu Cys Asp Val

CTT CCC TTG AAG CTG CTG GTG CCC CAG TGT CAC CAC GTG CTT GAC  362
Leu Pro Leu Lys Leu Leu Val Pro Gln Cys His His Val Leu Asp  118

GTC TAC TTC CCC CTC ACC ATC ACC TAC TTC CAG AGC CAG ATT AAT
Val Tyr Phe Pro Leu Thr Ile Thr Tyr Phe Gln Ser Gln Ile Asn

GCA AAG GCC ATC TGC CAG CAC CTG GGC CTG TGC CAA CCC GGG TCA  452
Ala Lys Ala Ile Cys Gln His Leu Gly Leu Cys Gln Pro Gly Ser  148

CCA GAG CCT CCG CTG GAC CCT CTG CCT GAC AAG CTG GTC CTC CCC
Pro Glu Pro Pro Leu Asp Pro Leu Pro Asp Lys Leu Val Leu Pro

ACA CTG CTG GGG GCC CTC CCA GCA AAG CCT GGG CCC CAC ACG CAG  542
Thr Leu Leu Gly Ala Leu Pro Ala Lys Pro Gly Pro His Thr Gln  178

GAT CTG TCG GCG CAG CCG TTC CCC ATC CCC CTG CCC TTG TGC TGG
Asp Leu Ser Ala Gln Arg Phe Pro Ile Pro Leu Pro Leu Cys Trp

CTC TGC AGG ACT CTC CTC AAG CGG ATC CAG GCC ATG ATT CCC AAG  632
Leu Cys Arg Thr Leu Leu Lys Arg Ile Gln Ala Met Ile Pro Lys  208

GGT GTC CTG GCC ATG GCT GTG GCA CAG GTG TGC CAC GTG GTA CCC
Gly Val Leu Ala Met Ala Val Ala Gln Val Cys His Val Val Pro

CTG GTG GTG GGC GGT ATC TGT CAG TGC CTG GCC GAA CGC TAC ACT  722
Leu Val Val Gly Gly Ile Cys Gln Cys Leu Ala Glu Arg Tyr Thr  238

GTC ATT CTG CTG GAG GTG CTA CTG GGC CAT GTG CTG CCC CAG CTG
Val Ile Leu Leu Glu Val Leu Leu Gly His Val Leu Pro Gln Leu

GTC TGT GGC CTT GTC CTC CGG TGC TCC AGC GTG GAC AGC ATT GGC  812
Val Cys Gly Leu Val Leu Arg Cys Ser Ser Val Asp Ser Ile Gly  268

CAA GTC CCG CCC ACT CTG GAA GCC CTG CCA GGG GAG TGG CTG CCG
Gln Val Pro Pro Thr Leu Glu Ala Leu Pro Gly Glu Trp Leu Pro

CAA GAC CCA GAG TGC CGC CTT TGC ATG TCT GTG ACC ACC CAG GCC  902
Gln Asp Pro Glu Cys Arg Leu Cys Met Ser Val Thr Thr Gln Ala  298

AGG AAC ATC AGT GAG CAG ACC AGG CCG CAG GCA GTG TAC CAC GCC
Arg Asn Ile Ser Glu Gln Thr Arg Pro Ala Ala Val Tyr His Ala
                ▲

TGC CTC AGC TCC CAG CTG GAC AAG CAA GAG TGT GAA CAA TTT GTG  992
Cys Leu Ser Ser Gln Leu Asp Lys Gln Glu Cys Glu Gln Phe Val  328

GCT GCA CAC GCC CCA GCT GCT GAG CCT GCT GTC CAG GGG TTG GGA
Ala Ala His Ala Pro Ala Ala Glu Pro Ala Val Gln Gly Leu Gly

TGC CCG CGC AAT CTG CCA GGC CCT GAG GGG CGT GTA GTG GCC ACG  1082
Cys Pro Arg Asn Leu Pro Gly Pro Glu Gly Arg Val Val Ala Thr  358

CTC AGC CCT CTC CAG TGC ATC CAA AGC CCT CAC TTC TGA GGACTCA
Leu Ser Pro Leu Gln Cys Ile Gln Ser Pr  His Phe End          370

GCTGTCCAGCCTGAATCACAGCCCAACACCAGAGCCACTTCTGTCCTGTCCCGGCAAGCA  1187
GCAAGAGCCGTGTGCCCCACAGCAGGTCTGCAGAGAGACCCCACAGCCTTCACTCCAGC  1246
AGAGCCACACGAGGCTCCCACAGCCTCCCAGGGGCCAGGCTGACCAAATACTGCCTCCC  1305
CACCCCCACCCCCACCTCACTCCAGAGACTGGCTTTACCGCTACTTGCTCACAGTCTAC  1364
GAAGTTCAAAATACAGAAGAGAGAGGCTGGTGCTGTGACCCAGCAGGTTGGCCACCGCC  1423
TGCAGTGCCGGCATGCCATGTGGGCACCAGTTTGAGTTCCAGCTGCTCCACTCCTGATC  1482
CAGCTCCCTCGTAACGCGCCTGGGAAAGCAACAGAAGATGGTCCAAGTGTTTGGACCCC  1541
TGCCACCCATGTGGAGATCCAGAAGAAGCTCCTGGCTCCTGGCTGGCCCAGCCCTGGCC  1592
ATTGCAGCCATTTGGGGAGTGAACCAGCAGACGGAAGAGCTCAATCTCTAACTCTTACA  1659
AATAAATAAAATAAATCTTTAAAAAATAAATACATCAAGG                      1693
```

mass of 8,500 Da, based on comparison with the bovine SP-B sequence (Olafson et al., 1987). This active fragment of rabbit SP-B is predominantly hydrophobic with an average pI of 8.43 and has the potential to form two amphipathic helices by residues 10-22 and 57-67 (Fig. 1.5.B, for detail see section 3.3). Three conserved cysteine motifs found in the human sequence (Glasser et al., 1987; Jacobs et al., 1987), each with the sequence $CX_2CX_{23\text{-}27}CX_{9\text{-}11}CX_{24}CX_5C$ (where X is any amino acid) are retained in the rabbit sequence. One potential glycosylation site (Asn-Ile-Ser) is observed at amino acids 301-303, near the C-terminal of the sequence.

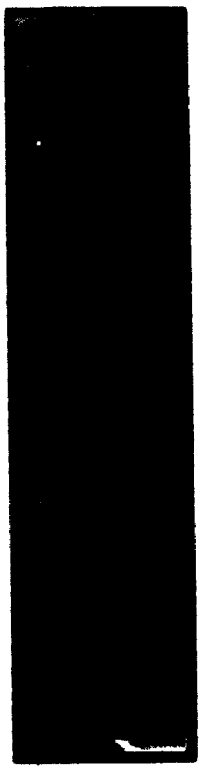
A distinct feature was found in the rabbit SP-B precursor sequence in a ten-amino acid fragment prior to the start site of the mature peptide (Phe_{186} as site +1). This segment (amino acids $_{175\text{-}185}$) has a sequence of Gly-Pro-His-Thr-Gln-Asp-Leu-Ser-Ala-Gln-Arg-(Phe_{186}) in the rabbit whereas in all other known species, including human, rat, dog and mouse, it is Gly-Pro-His-Thr-Gln-Asp-Leu-Ser-Glu-Gln-Gln-(Phe_{+1} or Leu_{+1}). The replacment of Gln_{-1} with Arg_{-1} and Glu_{-3} with Ala_{-3} in the rabbit would result in a positively charged residue (Arg^+) at -1 position and a non-polar amino acid (Ala) at -3 position as opposed to a non-charged polar residue (Gln) at -1 position and a negatively charged amino acid side chain (Glu^-) at -3 position in other species.

3.2.2. Coupled in vitro transcription-translation of the rabbit SP-B cDNA clone.

The cDNA clone was inserted into an in vitro transcription vector Bluescript.SK$^+$ downstream to a T7 promoter in both orientations. The T7 promoter can be used to generate specific transcripts with biological activity (Melton et al., 1984). Fig. 3.4.

Figure 3.4. Analysis of SP-B cDNA transcripts synthesized in vitro. *(A)* An autoradiogram of [α-^{35}S] labelled SP-B cDNA transcripts (SP-B cRNA) fractionated on urea-PAGE. The cRNAs were generated in vitro by RNA polymerase directed by T7 promoter upstream of the rabbit SP-B sequence in Bluescript SK$^+$ vector. The arrow indicates the position of the predicted full length transcripts. *(B)* Ethidium-bromide stained PAGE of SP-B cRNAs. Lane 1: SP-B cRNA. Lane 2: DNA molecular weight marker III (from bottom to top in kb: 0.56, 0.83, 0.94, 1.37, 1.59, 1.90, 1.98).

A.



← 1.7 kb

B.

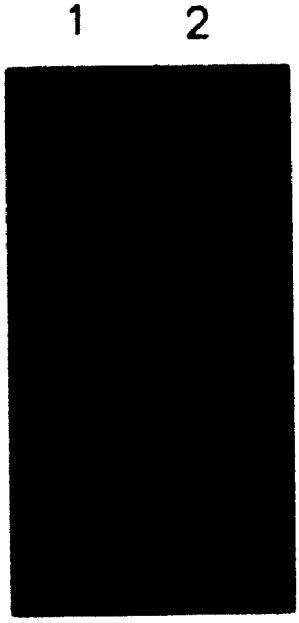
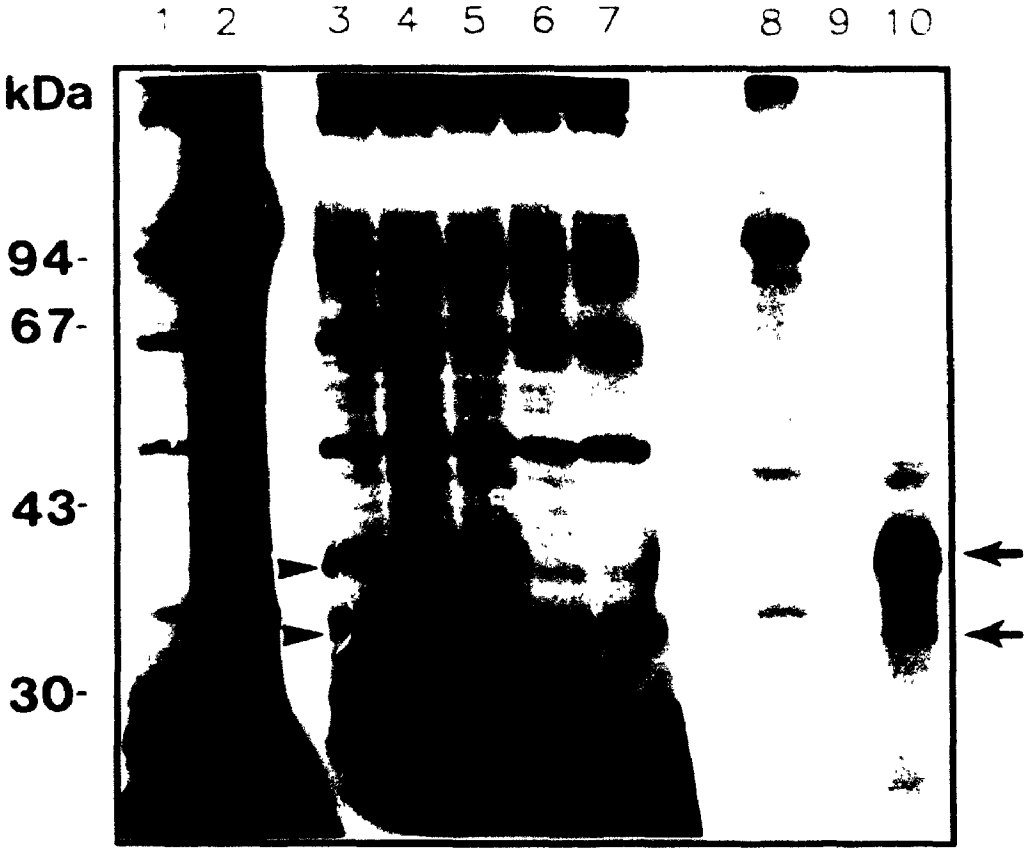
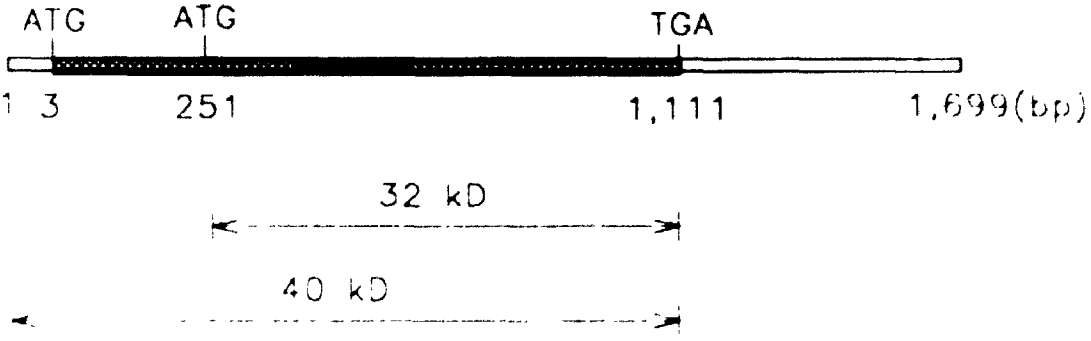


Figure 3.5. Analysis of in vitro transcription-translation products of rabbit SP-B cDNA. The upper panel shows a typical autoradiogram of [α-^{35}S]-labelled peptides fractionated on a 11 % urea-SDS-PAGE as described in section 2.5.3. The positions of the SP-B cRNA-dependent proteins are indicated by arrowheads. Lanes 1,2: translation products without exogenous RNA and with control Brome Mosaic Virus (BMV) mRNA. Lanes 3 to 5: translation products of the rabbit SP-B sense cRNA (0.5, 1.0 and 1.5 μg, respectively). Lanes 6,7: translation products of the anti-sense rabbit SP-B cRNA (0.5 and 1.0 μg, respectively). Immunological identification of the ^{35}S-labelled SP-B cDNA translation products is also shown. Rabbit anti-bovine SP-B antiserum #R10 was used in the immunoprecipitation assay (section 2.12.1). The resultant immunoprecipitated products of samples translated from BMV mRNA (lane 8), no exogenous RNA (lane 9) and rabbit SP-B sense cRNA (lane 10) are shown. The arrows indicate the positions of two SP-B peptides precipitated by the antisera. In the lower panel, a schematic illustration is shown for the two possible translation products predicted from the rabbit SP-B cDNA sequence. Two potential products could be translated starting at residue 3 or residue 251, yielding translation products with masses of 40 kDa or 32 kDa, respectively. A scheme of SP-B cDNA is shown by the bar, with untranslated regions drawn as open bars, the open reading frame as a striped area and the sequence for the active peptide as the darkened part.


1 2 3 4 5 6 7 8 9 10
kDa
94-
67-
43-
30-


ATG
ATG
TGA
1 3
251
1,111
1,699(bp)
32 kD
40 kD

shows that using T7 RNA polymerase, a cRNA of about 1700 nucleotides was generated. This cRNA was translated into proteins in vitro with a rabbit reticulocyte lysate system. The translation products of this SP-B clone were analyzed on urea-SDS-PAGE (Fig. 3.5.). Addition of 0.1 μg to 1.5 μg of the SP-B cRNA resulted in increasing amounts of proteins with apparent molecular masses of 40 kDa and 32 kDa (lanes 3-5). These proteins were observed when the sense strand (lanes 3-5) but not the antisense strand (lanes 6,7) of the cRNA was used as a template in the reaction, suggesting that the two bands corresponded to proteins encoded by the SP-B cDNA clone.

3.2.3. Immunoprecipitation of the translation products

An antiserum generated against the bovine SP-B peptide (R#10, kindly provided by Dr. Whitsett) was used to immunoprecipitate the proteins generated in vitro from the rabbit SP-B cDNA clone. Comparison of the deduced amino acid sequence of rabbit SP-B active fragment with that of mature bovine SP-B peptide revealed 82% identity. Lane 10 in Fig. 3.5. shows that the antiserum effectively immunoprecipitated rabbit SP-B proteins. It resulted primarily in the precipitation of a predicted major polypeptide of 40 kDa, which corresponds to the mass of a protein translated from the first methionine codon in the rabbit SP-B sequence and to the human SP-B precursor peptide (Jacobs et al., 1987; O'Reilly et al., 1988; Weaver et al., 1988). A similar product generated from fetal rabbit lung poly A^+ RNA could be immunoprecipitated as well (not shown). This indicated that the rabbit SP-B cDNA clone contains the entire sequence encoding for a 40 kDa precursor. Small amounts of a 32 kDa protein which may be translated from the

second ATG codon at nucleotide 251 were also precipitated.

3.3. Discussion

cDNA clones for the SP-B protein have, up to date, been isolated for human (Jacobs et al., 1987; Glasser et al., 1987), canine (Hawgood et al., 1987), rat (Emrie et al., 1989) and murine (D'Amore et al., 1991) lung. The nucleotide sequence encoding rabbit SP-B is similar to that in other species (Table 3.1). The coding sequences of SP-B cDNAs range in size from 1,111 bp in rabbit, 1,131 bp in mouse (D'Amore et al., 1991), 1,130 bp in rat (Emrie et al., 1989), 1,115 bp in dog (Hawgood et al., 1987) and 1,143 bp in human (Jacobs et al., 1987; Glasser et al., 1987). Comparison of the rabbit SP-B nucleotide sequence to those species revealed homologies ranging from 83% with the human, 77% with the mouse, rat and 80% with the dog (Table 3.1).

Direct RNA sequencing (Pilot-Matias et al., 1989) indicated that the 5'-untranslated region of human SP-B mRNA was very short, comprising only 14 nucleotides upstream from the initiator ATG. A similar spatial arrangement was also found in the promoter and exon 1 regions of the mouse SP-B gene (D'Amore et al., 1991). Two cytidine nucleotides located before the initiator ATG (nucleotide 1-2 in Fig. 3.3.) in the rabbit SP-B cDNA sequence are also present in the human (Jacobs et al., 1987), rat (Emrie et al., 1989) and mouse (D'Amore et al., 1991) sequences. Taken together, these results indicate that a short 5'-untranslated region may also exist in rabbit SP-B mRNA. The sizes of SP-B mRNA determined by Northern analysis vary from 1.9 kb in rabbit (Xu et al., 1989), 1.5 kb in rat (Emrie et al., 1989), 1.6 kb in mouse

Table 3.2. Homology of known SP-B sequences

Species	Percentage identify with rabbit SP-B		
	nucleotide sequence	primary translation product	mature protein sequence
human (1,2)	83	64	82
rat (3)	77	77	84
murine (4)	77	65	84
canine (5)	80	80	87
porcine (6)	N.D.	N.D.	82
bovine (7)	N.D.	N.D.	82

References:
1. *Glasser et al., 1987*
2. *Jacobs et al., 1987*
3. *Emrie et al., 1989*
4. *D'Amore et al., 1991*
5. *Hawgood et al., 1987*
6. *Curstedt et al., 1988*
7. *Olafson et al., 1987*

N.D.: not available

(D'Amore et al., 1991) and 2.0 kb in human (O'Reilly et al., 1988; Liley et al., 1989). The variation in SP-B mRNA size found among different species is primarily due to differences in length of the 3'-untranslated region. The mouse (410 bp) and rat (399 bp) 3'-noncoding regions are half the size of the human (823 bp), whereas the rabbit sequence is intermediate in size (577 bp). Little conservation of the 3'-untranslated sequences is seen among different mammalian species.

A single SP-B mRNA species is consistently detected on Northern blots for rabbit (Xu et al., 1989; also see chapter 4), human (Glasser et al., 1987), murine (D'Amore et al., 1991) and rat (Emrie et al., 1989). The murine and rat mRNAs have one and the human has two polyadenylation signals. Although four putative signals, three of which are overlapping and in tandem, are found in rabbit mRNA, they may not represent the complete situation. The rabbit SP-B cDNA examined in this study may not be a full length clone because there is a discrepancy between the sizes of this cDNA clone (1.7 kb) and that of SP-B mRNA detected from rabbit lung tissue (1.9 kb) by Northern analysis (see Fig. 4.1). This suggests there has been a loss of ~ 200 bp of 3' untranslated region in the cDNA clone as compared to the sequences obtained from the other species noted above.

There are several putative AU-rich regions in the 3' untranslated region of the human mRNA sequence (Jacobs et al., 1987), implying that SP-B mRNA levels could be controlled by stabilizing the message, as indicated by the study of O'Reilly and colleagues (1990), via a mechanism involving an AU-specific binding protein (section 1.4.1.2.). These AU-rich sequences in the human SP-B mRNA do not appear as

AUUUA motifs (Section 1.4.1.2) and they are not present in the rat, murine and canine SP-B sequences, which are believed to be full length. Although the rabbit SP-B cDNA clone is not complete, there are two AU-rich segments found at the end of 3' untranslated region (appearing as ATTT, TTTA). This could subject rabbit SP-B mRNA to pronounced post-transcriptional regulation as suggested by the studies presented in chapter 5.

The rabbit SP-B polypeptide sequence deduced from the cDNA shows high conservation among mammalian species. The rabbit preproprotein (Fig. 3.6) is consisting of 370 amino acids, with a molecular mass of ~40 kDa. It is therefore slightly larger than the canine preproprotein (368 amino acids) (Hawgood et al., 1987). but smaller than the murine peptide (377 amino acids) (D'Amore et al., 1991) and rat protein (376 amino acids) (Emrie et al., 1989). A putative N-linked glycosylation site within the C-terminal portion of the preproprotein is seen in the rabbit, murine, rat, and dog sequences. In humans, an additional site is seen in the N-terminal region (Jacobs et al., 1987). Significant protein sequence similarities at the preproprotein level are observed between the rabbit and the human (66%), canine (80.4%), rat (77%) and mouse (65%) (Table 3.1).

In the in vitro transcription-translation experiments presented in this study, two peptides with molecular masses of ~40 kDa and ~32 kDa were translated from the SP-B cDNA clone. The amounts of these two peptides apparent on SDS/PAGE increased in correspondence to the increasing amounts of SP-B cRNA (uncapped) added (Fig. 3.5). These two major peptides were immunoprecipitated by a polyclonal antiserum raised

Figure 3.6. Comparison of the deduced amino acid sequence of rabbit, dog, human and rat prepro-SP-B using PC Gene program. Dash symbols in the dog (**dog**), human (**hum**) and rat (**rat**) sequences indicate identity to the rabbit (**rab**) sequence. Gaps have been introduced within the sequences to achieve the greatest homology among the four species. The proposed mature protein is indicated in the rabbit sequence in bold and italicized type. An N-linked glycosylation consensus sequence (Asn-X-Ser) is indicated at position 301-303 of the rabbit sequence (#). The asterisks indicate the cysteine motif discussed in Sections 3.2 and 3.3.

```
rab   1 MAKSHLPPWLILLLLLPTLCGPGTAVWATSP  LACAQGPEFWCQSLEQALQCK
dog   1       -L -  ---------L- -A DW-APS----R--A-----------R
hum   1 --E---LQ----------------A-T--S  --------------------R
rat   1 ---L-- Q-  --------SL- -ATE -ASSPD-----K-------------

rab  52 ALGHCLQEVWGHVGADDLCQECQDIVNILTKMTKEAIFQDTIRKFLEHECD
dog  44 -----------NR ------------R------------SMV---------
hum  52 ----------------------E---H--N--A--------M-----Q--N
rat  49 ------------A--N------E---HL-------DA----------Q---

rab  103 VLPLKLLVPQCHHVLDVYFPLTITYFQSQINAKAICQHLGLCQPGSPEP
dog  94  -------T-----M-GT---VVVD-------P-I--K-----K--L---EQ
hum  103 -------M---NQ---D----V-D --N-TDSNG--M-----KSRQ---EQ
rat  100 I--------R-RQ-----L--V-D---G--KP----S-V---PL-QTK-EQ

rab  152                  PL DPLPDKLVLPTLLGALPAKPGPHTQDLSAQRFPI
dog  145 QESE              -S---L---I--E-P---QVT --------E-QL--
hum  153 EPGMSDPLPKPLRD--P---L------V-P---Q-R---------E-Q---
rat  151 KPEMLDAI          PN--LN-----A-P--FL-R---------E-QL--

                *  *                          *           * *
rab  189 PLPLCWLCRTLLKRIQAMIPKGVLAMAVAQVCHVVPLVVGGICQCLAERYT
dog  184 ---Y-------I-------------V--G-----------------G----
hum  204 ---Y-----A-I----------A--V-C----R-----A----------S
rat  194 ---F-------I--V--V-------V--S--------------------S

                            *     *
rab  239 VILLEVLLGHVLPQLVCGLVLRCSSVDSIGQVPPTLEALPGEWLPQDPECR
dog  234 -L--DA---RM-------------HE--AG    -A-AS--S--S--ESK-Q
hum  255 ----DT---RM------R------MD--A-PRS--      -----R-S--H
rat  245 ----DT---R-V------------TA-A--PAL-A--P-IEK-PL--T--H

                   #
rab  291 LCMSVTTQARNISEQTRPQAVYHACLSSQLDKQECEQFVAAHAPAAEPAV
dog  283 ---F-----G-H---AT---IRQ-----W--R-K-----EQ-M-RLQTLAS
hum  301 ---------G-S---AI---MLQ--VG-W--REK-K---EQ-T-QLLTL-P
rat  296 F-K--FN--W-T---AM---MHQ---RFW--R-K-----EQ-M-QLLAL-P

rab  341           QGLGCPRNLPGPEGRVVATLS-PLQCIQSPHF      370
dog  334 GGRDAHTTC-A--AC-T            -F- -----HI---      364
hum  352 RGWDAHTTC-A--              -CG-M-S-----H--DL      381
rat  347 RSQDAHTSC-AAVCEAPA             - ----F-T--L      376
```

against bovine mature SP-B. A molecular mass of ~40kDa has been assigned to the human SP-B precursor based on its cDNA sequence (Jacobs et al., 1987) and that detected by immunoprecipitation of [^{35}S]-labelled proteins from cultured H441 cells (O'Reilly et al., 1988) and human lung explants (Weaver et al., 1988; Whitsett et al., 1987a,c). The 32 kDa peptide produced from rabbit SP-B cRNA in vitro could be synthesized starting from the second methionine codon from the 5' end of the cDNA sequence. This codon is also present in the human sequence (Jacobs et al., 1987). However, the human SP-B cDNA produced only a single peptide of 40 kDa after immunoprecipitation, whether it was translated in vitro or after transfection into COS cells (Jacobs et al., 1987). Therefore, the production of the 32-kDa peptide from rabbit SP-B cRNA could reflect a non-specific initiating of protein synthesis from an uncapped RNA template.

As stated previously in Section 1.1.4.1, the 40-kDa SP-B precursor undergoes proteolytic processing to generate the 8-kDa active SP-B initiated with a Phe or Leu residue by being cleaved at both N- and C- termini of the active fragment. Recently, it has been shown that in humans this cleavage may occur intracellularly, most probably in multivesicular bodies (Voorhout et al., 1992) by cathepsin D-like proteases (Weaver et al., 1992). The ten-amino acid sequence (Section 3.2.1) located before the active SP-B (Phe_{+1} or Leu_{+1}) is completely conserved in human and other species including rat, dog and mouse (see Fig. 3.5). In the rabbit, however, the nature of the amino acids at positions -1 and -3 is different from all other known species ($Gln^{\pm}$ -> Arg^{+} and Glu^{-} -> Ala^{0}, respectively). This could imply (1) that these replacements would not affect the

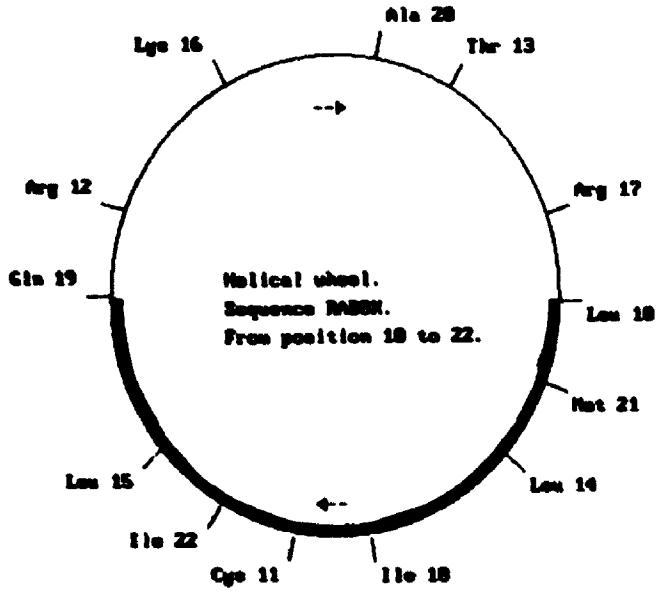
specificity of cathepsin D-like proteases resposible for producing the mature SP-B; (2) that other proteases could be involved in SP-B precursor processing in the rabbit; or (3) that the rabbit active SP-B protein does not start with Phe_{+1} as in human, bovine and porcine SP-B. The functional significance of these replacements in the rabbit precursor sequence requires further investigation.

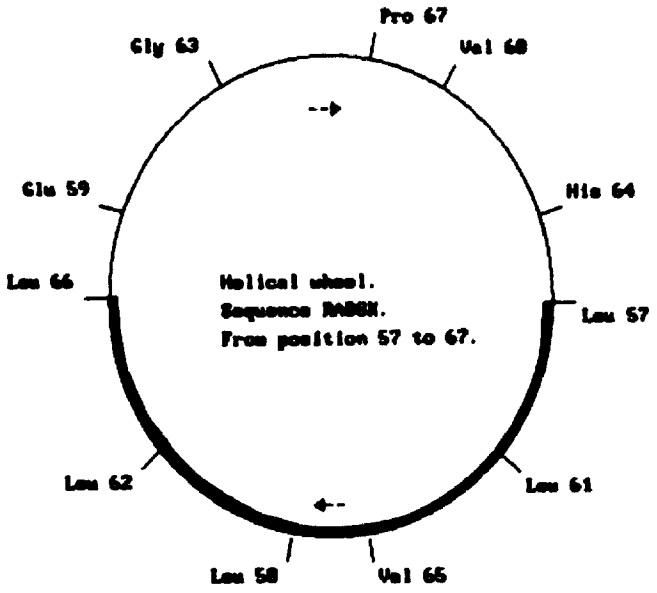
Considerable conservation of amino acid sequences is observed among mammalian SP-B active polypeptides (Fig. 3.6. and Table 3.1). The hydrophobic active SP-B in rabbit is similar to other active SP-B polypeptides: canine 87% (Hawgood et al., 1987); mouse 84% (D'Amore et al., 1991); porcine 82% (Curstedt et al., 1988); bovine 82% (Olafson et al., 1987) and human 82% (Jacobs et al., 1987; Glasser et al., 1987). Two regions of the rabbit active peptide (residues 10-22 and residues 57-67) resemble the corresponding regions found in other mammalian SP-B peptide in that they possess the potential to form amphipathic helices, as shown in the helical projections in Fig. 3.7. Models of these amphipathic helices suggest the hydrophobic portions of the helix (darkened circle, Fig, 3.7.) are likely to interact with acyl side chains of phospholipid, whereas the charged portion of the helix could interact with the polar head of the surfactant phospholipids, as discussed in Section 1.1.4.2.

One of the three cysteine motifs (section 3.2.1) was found within the SP-B active fragment sequence and the spacial arrangement of the cysteine residues in this motif were well conserved among different species. The conserved cysteine motif for rabbit active SP-B, shown in Fig. 3.7, consists of seven cysteine residues and their spatial distribution has the potential to form disulphide bonds within the active peptide monomer and

Figure 3.7. Sequence and structure of rabbit SP-B active peptide. Rabbit SP-B mature peptide sequence is shown at the top. Amphipathic regions within the peptide are in boldface. The cysteine motif discussed in the text is highlighted by asterisks below the sequence. Circle drawings depicting amphipathic helices are shown with darkened areas representing hydrophobic regions of helix.

FPIPLPLCWLCRTLLKRIQAMIPKGVLAMAVAQVCHVVPLVVCGICQCLAERYTVILLEVLLGHVLPQLVCGLVLRCSS





between monomers. A tertiary structure has been proposed for the porcine SP-B activepeptide (Johansson et al., 1991), based on HPLC size estimates of pepsin cleavage fragments from the purified peptide. It shows that three intrachain bridges link half-cystine residues 8 and 77, 11 and 71, and 35 and 46, respectively. This gives SP-B an appearance of three loops, a central big loop surrounded by two smaller ones. In the major form of SP-B, the remaining half-cystine, Cys-48, is probably interchain-linked to its counterpart in another molecule, compatible with the existence of dimeric molecules. Conservation of this cysteine motif in rabbit SP-B sequence suggests a possibility of the peptide to form a similar tertiary structure for this species. In contrast, the active peptide of bovine SP-B differs from the others because it lacks the second conserved cysteine residue in the motif (Olafson et al., 1987).

Two other conserved cysteine motifs are seen in the N-terminus starting at residue 70 and in the C-terminus starting at residue 289 in the rabbit preproprotein. The role of these two cysteine motifs in the proper folding of SP-B protein is not clear.

In summary, the rabbit SP-B mRNA sequence as revealed by cDNA sequence analysis is similar to that reported for other species. Comparison with other known mature SP-B sequences indicates that rabbit SP-B mature peptide could form the secondary and tertiary structures proposed for porcine SP-B peptide. The 3' AU-rich regions present in rabbit and human SP-B mRNA sequences but not in those of canine, rat and murine may imply species-specific mechanisms for regulating SP-B mRNA levels among these species.

CHAPTER 4 - EXAMINATION OF RELATIVE TRANSCRIPTION RATES OF THE SP-B GENE AND SP-B STEADY-STATE mRNA LEVELS IN DEVELOPING RABBIT LUNG

4.1. Introduction

The discovery that the lungs of infants who died of Hyaline Membrane Disease (HMD) were deficient in surfactant (Avery and Mead, 1959) led to numerous studies on the relationship between development of the surfactant system in the lung and RDS. These investigations included animal studies on the ontogeny of surfactant components and analysis of the phospholipid composition of human amniotic fluid for the prediction of HMD (Robertson et al., 1992), as well as attempts to prevent HMD by accelerating lung maturation in utero with maternal administration of certain hormones or agents (Ballard, 1989, 1992; Post and van Golde, 1988; Gross, 1990; Post, 1992). It was observed in ontogenic studies that surfactant components do not accumulate in the lung until late in gestation (the last 20% of gestation), explaining the high risk of HMD in preterm infants. This has led to the development of surfactant replacement therapy by instillation of extracts of purified bovine or porcine surfactant or artificial surfactant into preterm infant trachea (Jobe & Ikegami, 1987). Examination of the lecithin/sphingomyelin (L/S) ratio and the presence of phosphatidylglycerol in amniotic fluid during lung development has revealed that these two parameters are related to the stage of lung maturation. Low L/S ratio will indicate less maturity in the lung (Gluck, 1973). On the other hand, phosphatidylglycerol content increases in amniotic fluid as

the lung becomes mature. Therefore, measurement of L/S ratio and PG has become routine for prediction of the risk of HMD and for monitoring regimens involving antenatal hormonal administration to accelerate lung maturation (see below).

Based on the knowledge that glucocorticoids enhance fetal lung maturation and surfactant phospholipid production in animal models in vivo and in vitro (for review see Rooney, 1985; see also Section 1.2.2.1.), clinical studies have been conducted which demonstrated a decrease in the incidence and severity of respiratory distress after maternal administration of glucocorticoids (Collaborative group, 1981; Ballard et al.,1980). However, both animal and human studies suggested that the effects of glucocorticoids on fetal lung maturation are dependent, in part, on the gestational age when given. Prevention of RDS by glucocorticoids is more effective in infants between 30 to 34 weeks of gestation age (Collaborative group, 1981). Therefore, it is important to study the regulation of surfactant synthesis during fetal lung development, in order to further elucidate the mechanisms by which lung tissue responds to glucocorticoids and other hormones or agents during different stages of maturation. This could provide strategies for surfactant treatment. It was the aim of this study to explore in depth the regulation of one of the surfactant proteins, SP-B, in fetal rabbit lung during development.

While this work was in progress, the developmental expression of SP-B mRNA in human (Liley et al., 1989; Whitsett et al., 1987a) and rat (Schellhase et al., 1989) lung was reported. In human fetal lung tissue, initiation of expression of the gene encoding SP-B is detected at an early time in development, approximately around the

middle of the pseudoglandular phase (13 or 16 weeks of gestation). This is many weeks prior to the initiation of surfactant phospholipid and SP-A production which occur after 20 weeks gestation (see also Sections 6.1. and 6.3.). In agreement with these observations, SP-B protein levels in humans were undetectable or low during this same period of gestation (before 20 weeks of gestation) (Whitsett et al., 1987a). Similarly, in rat SP-B mRNA (Schellhase et al., 1989) and protein (Shimizu et al., 1991) were first detected in fetal lung tissue at day 18 of gestation, which is the end of pseudoglandular phase of lung development. Durham et al. (1993) and Connelly in this laboratory (1991) also reported that in the rabbit SP-B mRNA was readily detectable before SP-A mRNA.

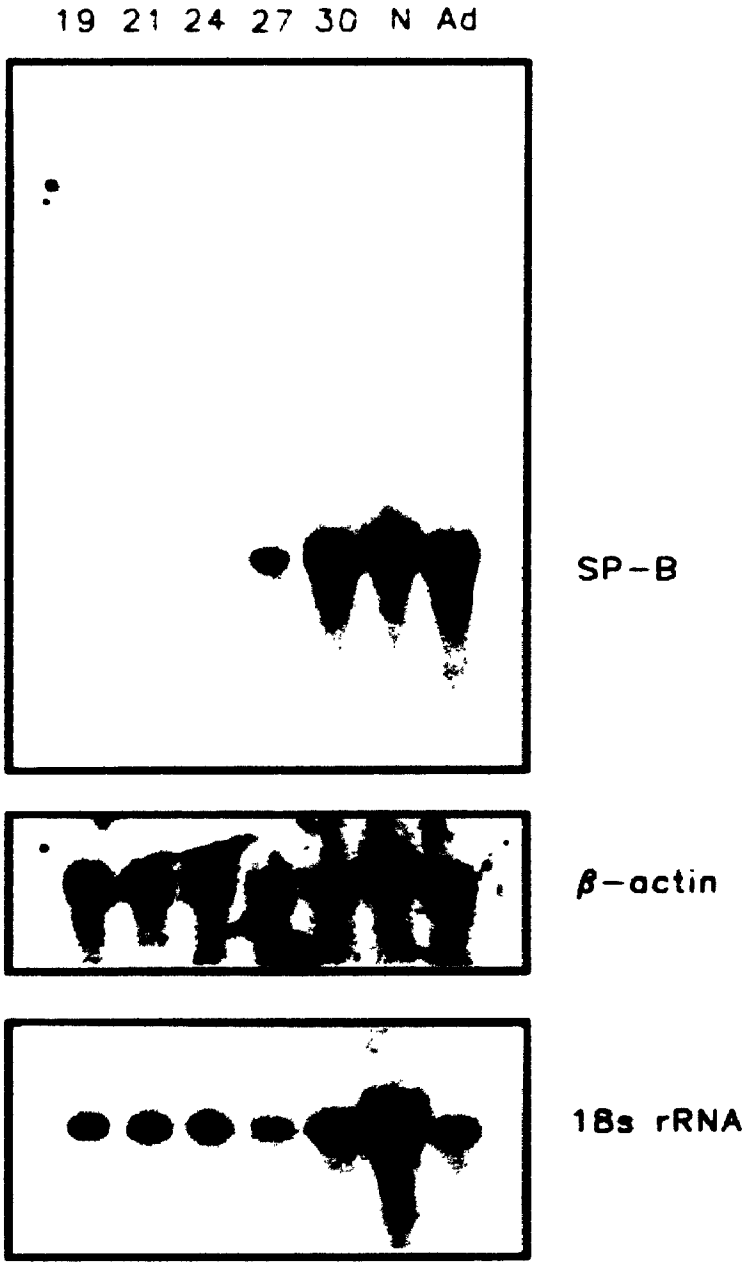
Although the ontogeny of SP-B has been examined at the mRNA and protein levels, transcriptional activity of the SP-B gene during fetal lung development has not been reported for any species. In this study we attempted to examine SP-B gene expression in fetal rabbit lung at both transcriptional and post-transcriptional levels in order to elucidate the mechanisms by which SP-B mRNA levels are regulated in vivo. The results obtained in these studies also serve as a reference point for in vitro studies presented in Chapter 5.

4.2. Results

4.2.1. Determination of SP-B mRNA steady-state levels.

Total RNA isolated from lung tissue of 19, 21, 24, 27 and 30 day gestational age fetal rabbits and from neonates (3 days) was subjected to Northern blot analysis (Fig. 4.1.) by hybridization with a ^{32}P-labelled rabbit SP-B cDNA probe (Chapter 3). The

Figure 4.1. Alterations in SP-B gene transcription and mRNA levels in rabbit lung during perinatal development. *Upper panel:* Representative autoradiogram of Northern analysis of SP-B mRNA levels. Total RNA (10 μg) isolated from lung tissues of 19 to 30-days gestational age fetuses (19-30), neonatal 1 day (N) or adult (Ad) rabbits was fractionated, transferred and hybridized with a ^{32}P-labelled SP-B cDNA probe as described in Section 2.6. The blot was stripped then reprobed with a ^{32}P-labelled rabbit 18s cDNA probe or mouse β-actin probe. The positions of the RNAs are indicated. *Lower panel:* Representative autoradiograms of nuclear run-on analysis of transcriptional activity of the SP-B gene. Nuclei isolated from rabbit lungs of gestational 22 to 30 days (22-30), neonatal 1 day (N1D) and adult (Ad) animals were used. The ^{32}P-SP-B nuclear transcripts were detected by a rabbit SP-B cDNA immobilized on a filter. A mouse β-actin cDNA was also included on the filter as a reference message.


19 21 24 27 30 N Ad
SP-B
β-actin
18s rRNA

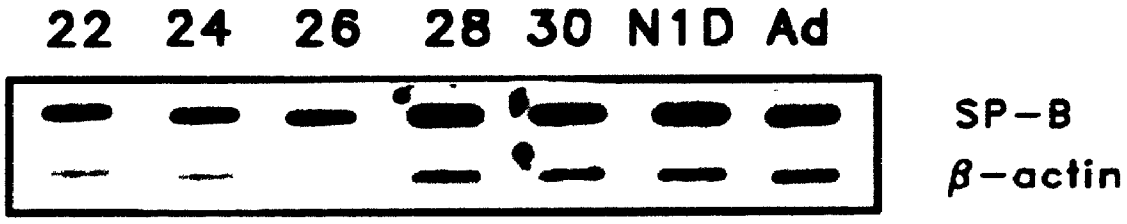
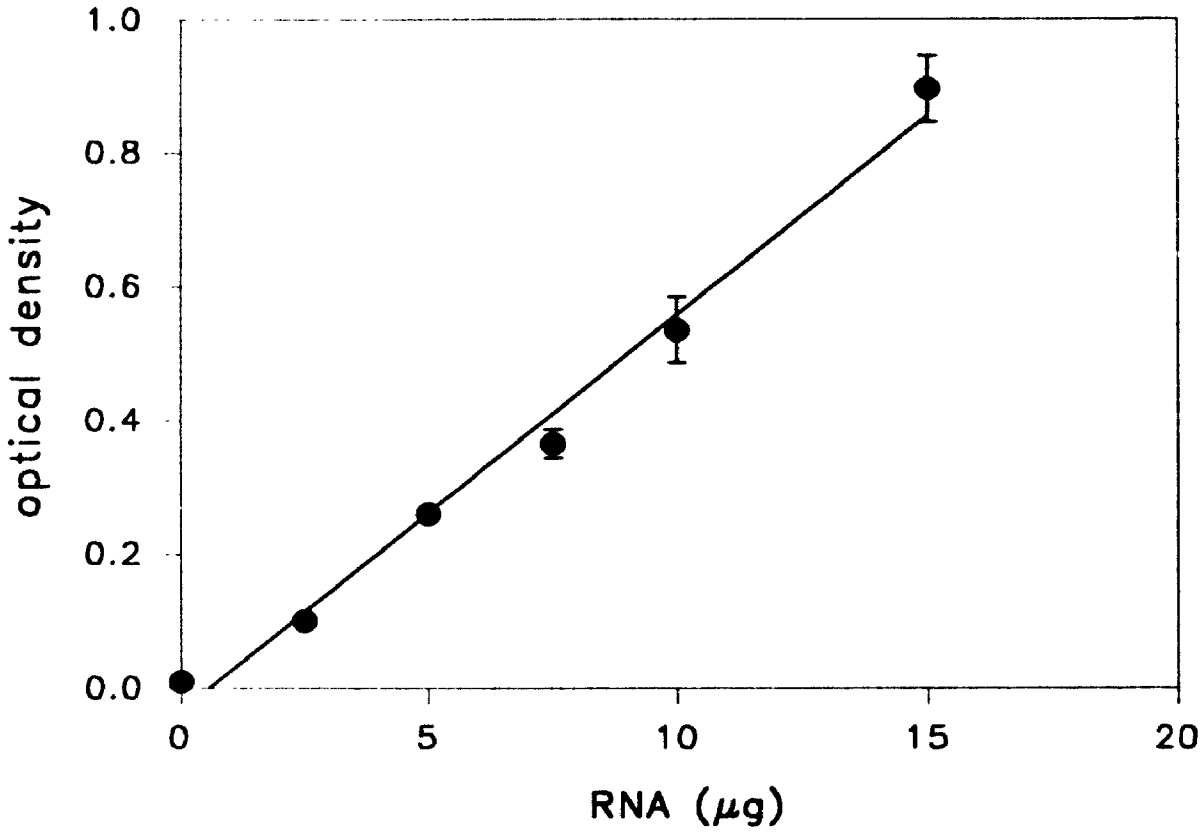

22 24 26 28 30 N1D Ad
SP-B
β-actin

Figure 4.2. Slot blot analysis for quantifying SP-B mRNA levels in rabbit lung tissue. Increasing amounts of total RNA isolated from rabbit lungs of 30-days gestations were denatured and blotted in duplicate onto a nitrocellulose membrane as described in section 2.6.2. The filter was hybridized to a ^{32}P-labelled rabbit SP-B cDNA probe. The optical density of the hybridized SP-B mRNA of the autoradiogram is plotted on the Y-axis and the amount of cytoplasmic RNA loaded in the slot is plotted on the X axis. The optical densities obtained with an equal amount of total liver RNA were consistently lower than 5% of the positive signals (not shown).


1.0
0.8
0.6
0.4
0.2
0.0
optical density
0
5
10
15
20
RNA (μg)

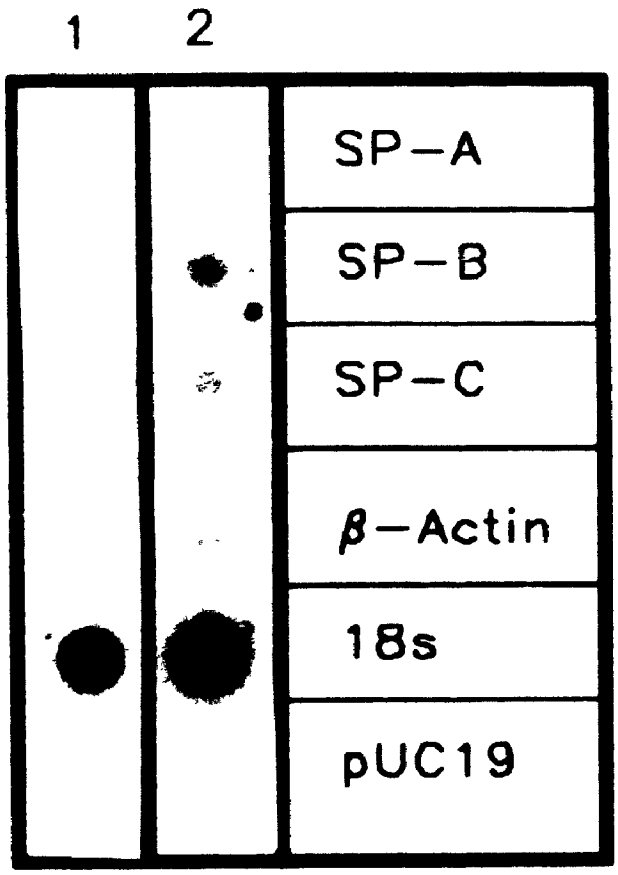
presence of a single RNA species of 1.9 kb was observed in lung tissue at 27 days gestation. The relative proportion of SP-B mRNA increased thereafter until it reached a peak on 30 days gestation (term 31 days) but declined slightly after birth compared to 30 days gestation. This pattern of changes in SP-B mRNA levels was specific since it was not observed with 18s rRNA or β-actin mRNA.

In order to better quantify SP-B mRNA levels, total RNA from lung tissues of 22, 24, 26, 28, 30 days gestation, neonatal and adult rabbits was also subjected to slot blot analysis followed by laser densitometry of the autoradiograms. The same SP-B cDNA used for the Northern analysis was used as the probe. The amount of total RNA (5 μg) immobilized on nitrocellulose membrane was optimized so that the signal produced on the autoradiogram fell within the linear range for densitometry (Fig. 4.2). The results, presented as values relative to that of adult lung (hatched bars in Fig. 4.6.), showed that SP-B mRNA could be detected as early as 22 days gestation instead of 27 days as shown in Northern analysis (Fig. 4.1.). On 30-days gestation, SP-B mRNA levels were about 1.7-fold higher than that of adult lung ($P > 0.05$). The levels determined in neonatal day 1 lung were not significantly different from the levels in adults. RNA samples from two adult rabbits were used to normalize the data.

4.2.2. Characterization of nuclear run-on assays

Since the accumulation of a specific mRNA could be regulated by either (1) an increase in its rate of synthesis or (2) a decrease in its rate of degradation, it was of interest to measure these parameters directly with regards to SP-B mRNA during

Figure 4.3. Lung-specific production of surfactant protein mRNAs. Nuclei isolated from liver (lane 1) and lung (lane 2) tissues from 30-days gestation rabbits were allowed to transcribe their nascent RNAs in vitro (section 2.9.2). The [^{32}P] RNA transcripts (1 X 10^6 cpm/1.5 ml) were hybridized to the indicated cDNAs (2.5 pmole each cDNA) immobilized on nitrocellulose membrane. A typical autoradiogram is shown. The lung-specific nature of transcription of the surfactant protein genes remained apparent after longer exposure.


1
2
SP-A
SP-B
SP-C
β-Actin
18s
pUC19

perinatal development. To address the first question, a technique called nuclear transcription run-on assays was adopted. It basically involves allowing isolated nuclei to transcribe their nascent RNA in vitro in the presence of [^{32}P]UTP or [^{32}P]GTP and hybridizing the radioactive RNA produced to filter-bound recombinant DNA containing coding sequence from the gene of interest, in our case, the SP-B gene. After intensive washing in NaCl/SDS then RNase treatment, the filter can either be subjected to autoradiography or treated with alkali to release the ^{32}P-RNA which is then counted in scintillation fluid.

Normally, to reduce nonspecific binding to a minimum, filters with a high capacity for binding specific RNAs should be prepared and their specificity determined. In our case, the specificity of SP-B cDNA was determined by (1) observing that only one band was detected on a Northern blot when using the SP-B cDNA insert as a probe; (2) optimizing that the hybridization stringency, washing and RNase conditions such that less than 5% signal is produced by the wild type plasmid DNA sequence; and (3) confirming that [^{32}P]RNA transcribed from adult liver nuclei failed to hybridize to SP-B cDNA but not to 18s cDNA or β-actin cDNA, since liver does not express surfactant proteins (Fig. 4.3).

An internal control was included with each assay consisting of [^{32}P]RNA transcribed from adult lung nuclei. All the data were expressed as values relative to that of adult lung nuclei. A coefficient of variation (CV) of 15.7% was obtained for total incorporation of [^{32}P]UTP or GTP in adult nuclei from same preparation used in separate assays, and 20.5% for adult nuclei prepared separately.

Figure 4.4. The rate of SP-B mRNA synthesis in isolated nuclei as a function of incubation time. Lung nuclei were isolated from 30 days gestational rabbits. The nuclei were allowed to transcribe at 30°C for the times indicated using the standard conditions described in section 2.9.2. At 7, 14, 34 and 45 min, the total incorporation of [^{32}P] UTP *(line)* was determined and plotted on the Y-axis as a percentage of acid precipitated material to the total cpm of the aliquot. The rate of SP-B mRNA synthesis was also measured as described in Section 2.9.2. and Fig. 4.3. Optical densities obtained from densitometry of the autoradiogram are plotted in *bar diagrams*. Data in each plot are means of two determinations.

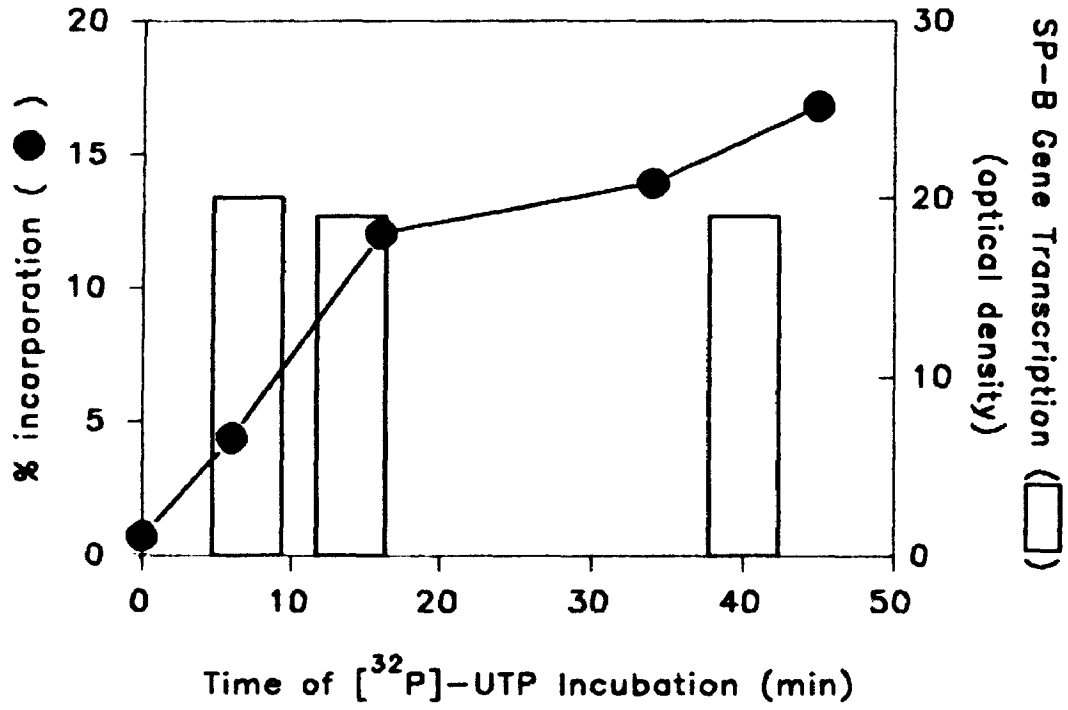
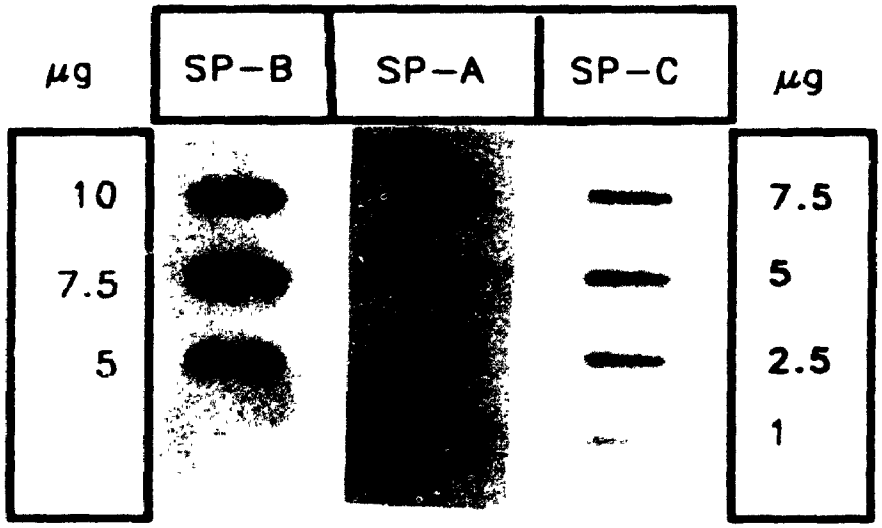

20
15
10
5
0
% incorporation (●)
30
20
10
0
SP-B Gene Transcription (□)
(optical density)
0
10
20
30
40
50
Time of [32P]-UTP Incubation (min)

Figure 4.5. Determination of the optimal amounts of SP-A, SP-B and SP-C cDNA required on nitrocellulose membranes for the nuclear run-on assays. Filters with various amounts of the three cDNAs were hybridized with [^{32}P]RNA transcripts synthesized by isolated nuclei from lung tissue of gestational 30 day rabbits. The autoradiograms were obtained as in Fig. 4.3. Approximately 2.6 pmoles or 7.5 μg of SP-B cDNA and equimolar amounts of SP-A (7 μg) and SP-C (6 μg) were found to be sufficient for use in the run-on assays.


μg
SP-B
SP-A
SP-C
μg
10
7.5
5
7.5
5
2.5
1

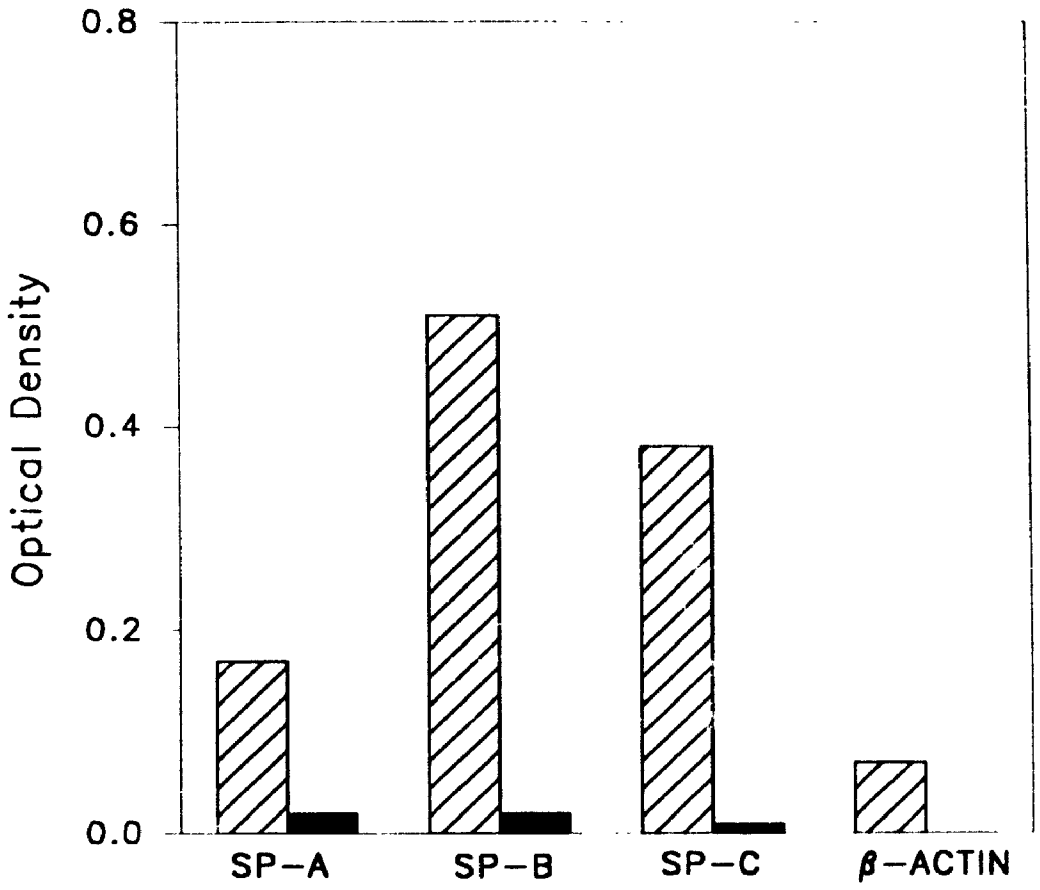
The transcription assays were performed for 7,14 and 40 mins with isolated adult nuclei. Figure 4.4 shows that more than 50% of total [^{32}P]UTP incorporation occurred within the first 15 min. The relative rate of SP-B mRNA synthesis decreased slightly after 7 min and then remained basically constant thereafter. This indicates that most of the SP-B RNA transcribed remains large enough to form stable hybrids even after 40 min of incubation. This incubation time of 40 min was used routinely.

In addition, as shown in Fig. 4.5, the amounts of the DNA of interest immobilized on the nitrocellulose membrane was determined to be in excess of that required to measure the corresponding [^{32}P]RNA transcripts.

4.2.3. Messenger RNA is transcribed from the SP-B gene by RNA polymerase II

When nuclei were transcribed in the presence of α-amanitin (1 μg/ml), the total incorporation of [^{32}P]UTP was inhibited by about 50% (data not shown) and the specific transcription of the SP-B gene was blocked by more than 95% in nuclei of either 30 days gestation lung or adult lung, as shown in Fig. 4.7. Since α-amanitin has been shown to be a specific inhibitor of RNA polymerase II at this concentration (Kedinger et al., 1970), we concluded that the SP-B gene, as are the SP-A and SP-C genes, is normally transcribed by RNA polymerase II. This result is consistent with that published recently by O'Reilly et al. (1991) showing that SP-B mRNA synthesis in a human lung tumour cell line is inhibited by α-amanitin.

Figure 4.6. The effects of α-amanitin on the transcription of SP-A, SP-B, SP-C and ß-actin mRNAs. Nuclei isolated from adult rabbit lung were allowed to incorporate [^{32}P]UTP (Fig. 4.4) in the presence *(solid bars)* or absence *(hatched bars)* of α-amanitin (1 μg/ml). The ^{32}P transcripts were hybridized with filters containing immobilized SP-A, SP-B, SP-C and ß-actin DNA. Optical densities obtained upon densitometry of the autoradiogram are plotted.

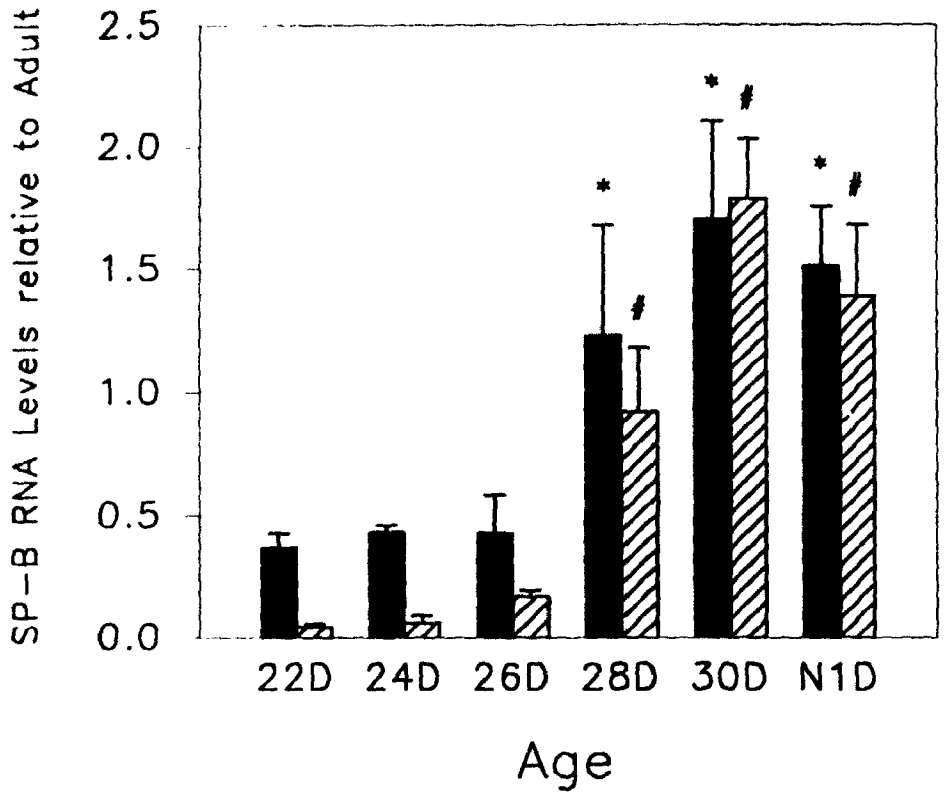

0.8
0.6
0.4
0.2
0.0
Optical Density
SP−A
SP−B
SP−C
β−ACTIN

4.2.4. Measurement of relative rates of SP-B gene transcription during pulmonary maturation

To determine relative transcription rates of the SP-B gene during the perinatal period, nuclear transcriptional run-on assays (section 4.2.2) were performed with nuclei isolated from 22, 24, 26, 28, 30 day fetal, neonatal day 1 and adult rabbit lungs. A typical autoradiogram is shown in Fig. 4.1 (B). The results obtained after densitometry of the autoradiograph are presented in Fig. 4.7 as values relative to that of adult lung (normalized with two adult samples). Transcriptional activity of the SP-B gene was readily detected as early as 22-days gestation, with a level about half of that of the adult. A marked increase, however, was not observed until 26 to 28-days gestation. Transcriptional activity of SP-B gene reached 1.7-fold adult levels by 30 day of gestation and fell slightly on neonatal day 1. Comparing the transcription rates to the mRNA levels detected by Northern and slot blot analysis (Fig. 4.7), the changes in SP-B gene transcription showed a close parallel relationship with that of SP-B mRNA accumulation during this perinatal period, except at the early stage, 22-24 days of gestation, where relatively higher levels of the SP-B gene transcription were observed. This is suggestive that SP-B mRNA levels during the later stage could be primarily controlled in vivo at the transcriptional level.

It should be noted that the pattern of changes in SP-B gene activity were specific since the transcription rates of the β-actin gene, although also accelerated in development during the same period as the SP-B gene, continued to increase in nuclei isolated from 28 days, 30 days gestation and neonatal and adult lungs. The overall transcription

Figure 4.7. Comparison of SP-B gene transcriptional activity and SP-B mRNA levels in rabbit lung during development. SP-B mRNA levels were quantified by slot blot analysis of the cytoplasmic RNA isolated from rabbit lung tissue of indicated ages. The autoradiograms obtained from both nuclear run-on *(solid bars)* and the slot blotting *(hatched bars)* experiments were subjected to densitometry. The data are nomalized with 18s rRNA signals and presented relative to adult values (=1). Data are means±S.E. for three or four separate litters. * or # indicates a p value of less than 0.05 compared to the corresponding 22-day values.


SP-B RNA Levels relative to Adult
2.5
2.0
1.5
1.0
0.5
0.0
22D
24D
26D
28D
30D
N1D
Age
*
#

activities of nuclei isolated from lung at different ages were similar and the DNA contents measured by the ethidium bromide-fluorescence method remained constant in lungs of all ages tested (data not shown).

4.2.5. Determination of SP-B mRNA turnover rates.

To further investigate whether the SP-B mRNA levels were mainly controlled at the transcriptional level during perinatal period, the relative turnover rates of SP-B mRNA were measured in lungs of gestational age 26-day and 30-day, neonatal day 1 and adult rabbits. The approach used was to expose lung explant culture to actinomycin D, an antibiotic which specifically blocks activity of RNA polymerases, for up to 48 h followed by measurement of pre-existing SP-B mRNA molecules by solution hybridization assay (Fig. 4.12) and slot blot analysis (Fig. 4.2).

The efficiency of actinomycin D in blocking the transcription in lung explants was determined by ^{3}H-uridine incorporation (Fig. 4.8). It revealed that as low as 0.75 μM of the drug can effectively block over 90% of the total transcriptional activity in 24 h in the lung tissue. Two different concentrations of actinomycin D, 1 μM and 5 μM, were used in the study. The cytotoxicity of the inhibitor was also examined using light microscopy (Fig. 4.9). With 1 μM of actinomycin D (panel 2), lung tissue showed somewhat improved intactness than with 5 μM (panel 3) of the drug. However, the half-life obtained for SP-B mRNA using 1 μM or 5 μM actinomycin D were not significantly different. The effect of actinomycin D on SP-B mRNA levels was specific since 18s rRNA was not significantly affected, as shown by the Northern blot in Fig. 4.10.

Figure 4.8. Effect of various concentrations of actinomycin D on the incorporation of ^{3}H-uridine into total cellular RNA in explants of adult rabbit lung incubated for 24 h. The values are presented as a percentage of control value (means$\pm$S.D, n=3). As low as 0.75 μM of actinomycin D was sufficient to reduce the transcription in the explants by 90%.

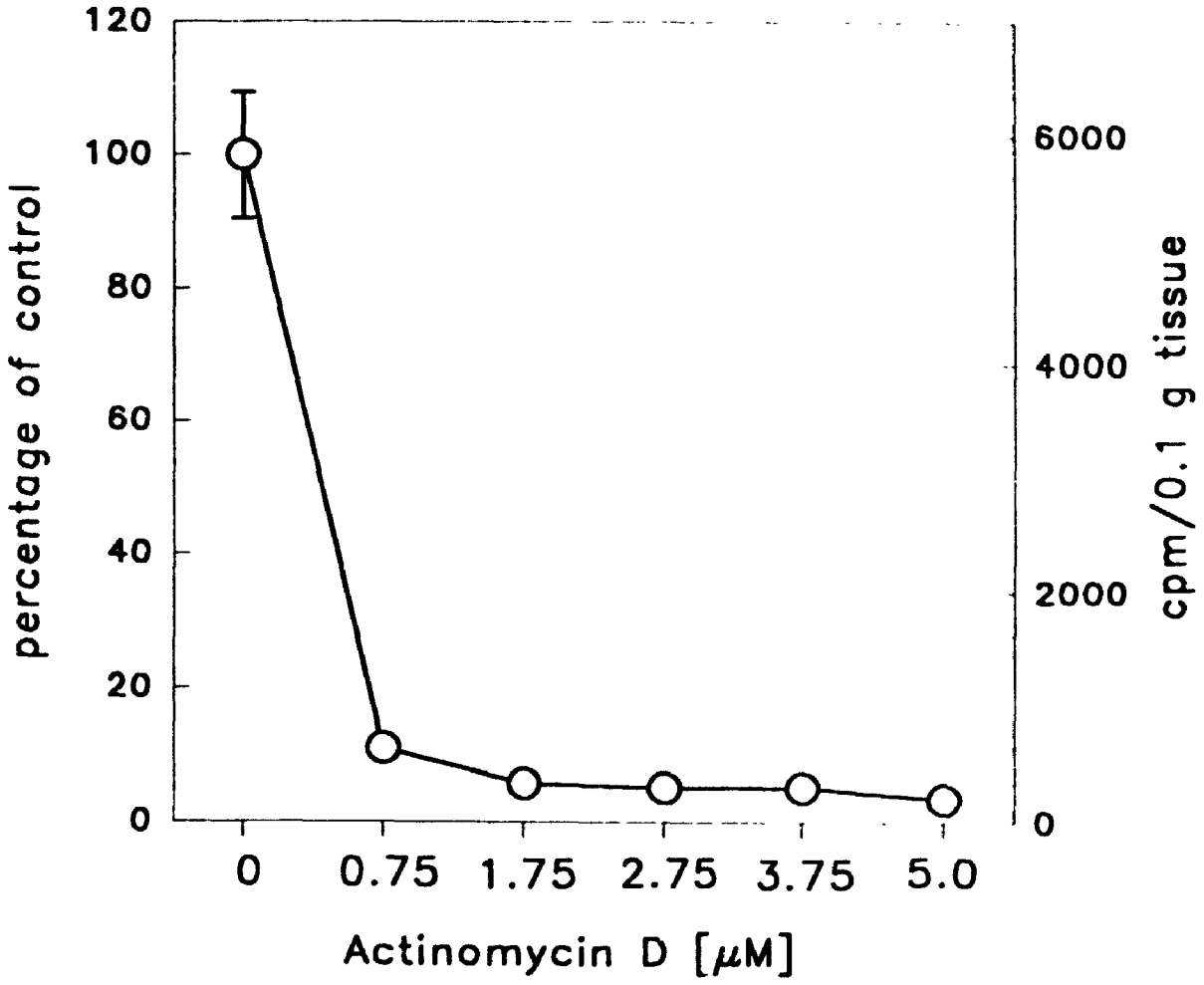

120
100
80
60
40
20
0
percentage of control
6000
4000
2000
0
cpm/0.1 g tissue
0
0.75
1.75
2.75
3.75
5.0
Actinomycin D [μM]

Figure 4.9. Effects of 1 μM and 5 μM actinomycin D on the morphological appearance of rabbit lung explants. Light micrographs (40X) of 26 day rabbit lung explants after two days of culture in control medium (*1*), in medium containing 5 μM (*2*) or 1 μM (*3*) actinomycin D. Cell death was evident in explants in the present of 5 μM actinomycin D. Cytotoxicity was visibly less in lung explants incubated with 1 μM actinomycin D than those with 5 μM actinomycin D.

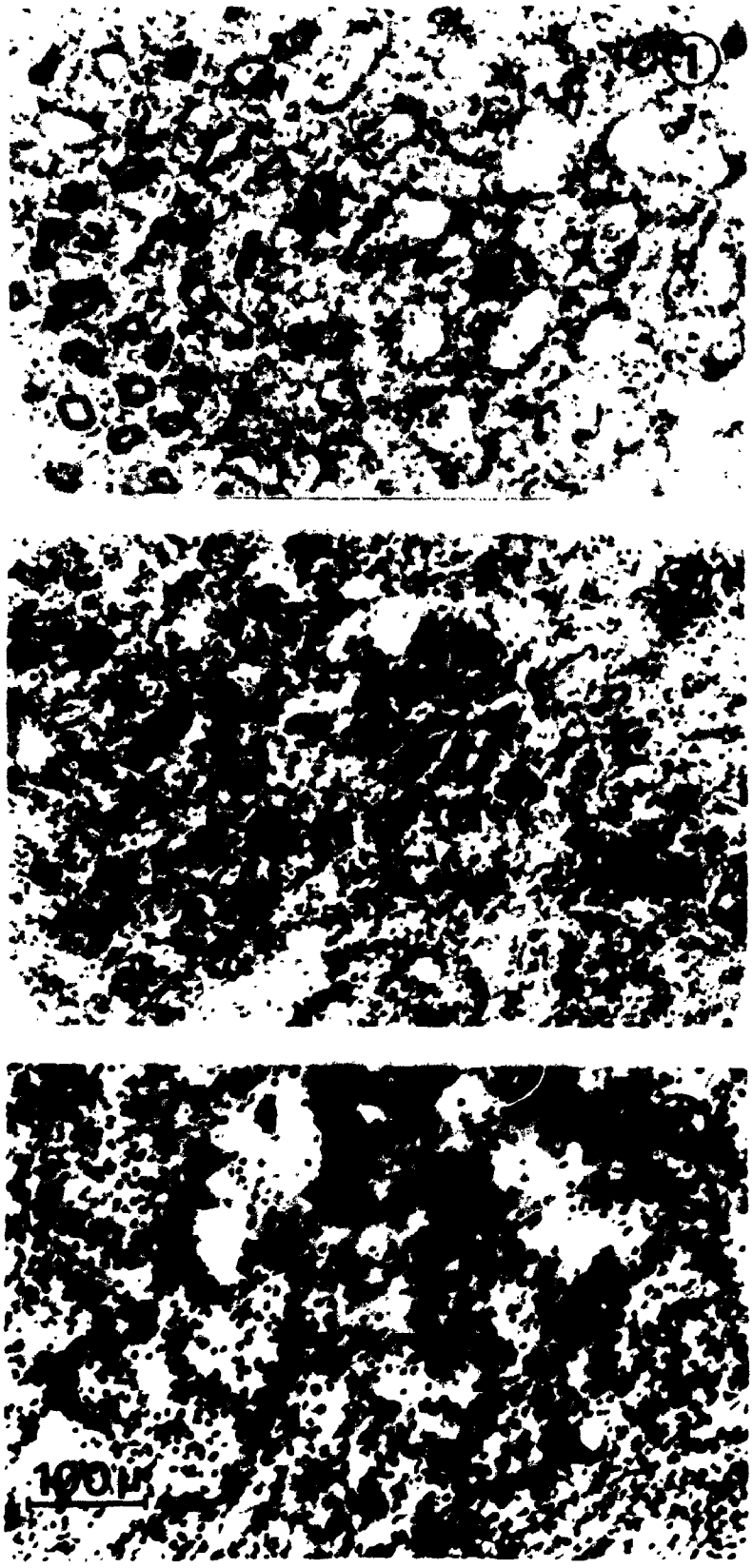
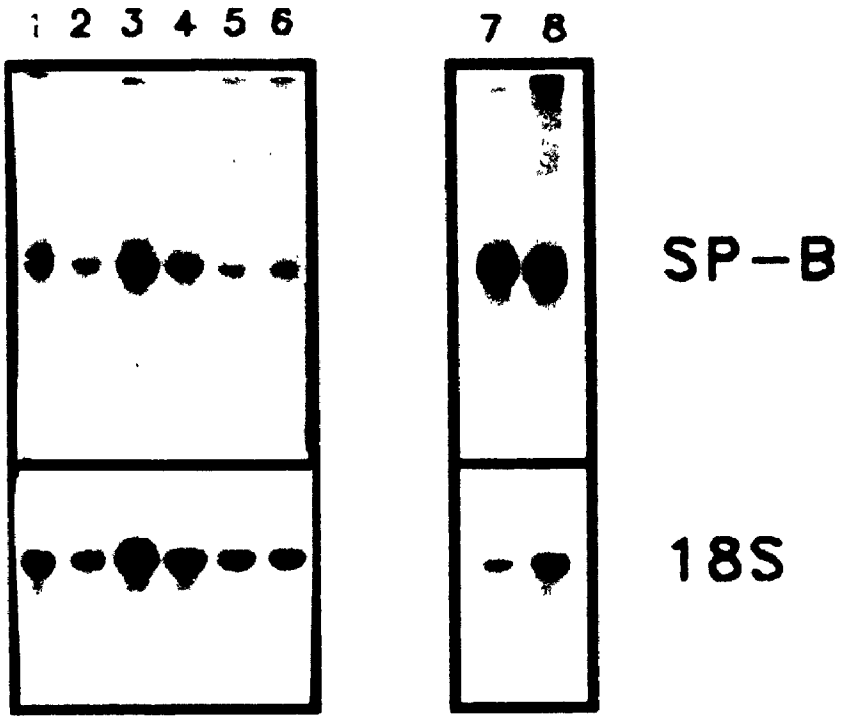

100μ

Figure 4.10. Northern blot analysis of SP-B mRNA isolated from 26-day lung explants cultured in the presence and absence of 1 μM actinomycin D. The inhibitor was added at the beginning of culture. Tissues were harvested after 6, 18, 30, 42, 48 and 48 hours with the inhibitor (lanes 1-6, respectively), or without actinomycin D for 30 and 48 hours(lanes 7 and 8). RNA was extracted as in Section 2.3.2. Ten micrograms RNA were fractionated, transferred onto a Nytran membrane and hybridized with ^{32}P-labelled SP-B probe. The blot was then stripped and hybridized with a [^{32}P] 18S probe.


1 2 3 4 5 6
7 8
SP-B
18S

As shown in Fig. 4.12., the relative turnover rates of SP-B mRNA measured in lungs of different ages were qualitatively similar to one another, with the half-life of the message being 32.7±4.6 h, 24.4±1.9 h, 26.5±3.6 h and 31.3±5.1 h for 26-day, 30-day gestations, neonatal 1 day and adult rabbits, respectively (means±S.E.M., n=4 for each age). Statistic analysis using one way ANOVA showed no significant difference among these values.

4.3. Discussion

In this chapter, the ontogeny of SP-B mRNA accumulation and the relative rates of SP-B gene transcription were examined in developing rabbit lung. The developmental pattern for SP-B gene transcription in the present study was the first reported.

SP-B mRNA levels during lung development have been examined in rat (Schellhase et al., 1989), early human fetal (Liley et al., 1989), as well as in rabbit lungs (Durham et al., 1993; Connelly et al., 1991). SP-B mRNA was first detected on 18/22 (gestation/term) days gestation in rat lung; around 13 weeks gestation (term=40 weeks) in the human. The times reported for the first detection of SP-B mRNA in rabbit lung are not in full agreement. Durham and colleagues observed, by Northern analysis, that SP-B mRNA was readily detected at 24 days gestation (term=31 days), earlier than we observed in the present study using the same method. Wohlford-Lenane et al. (1992b) from the same group reported that, using in situ hybridization, SP-B mRNA was not present in 19 and 21 days fetal lung but could be detected on 24 days gestation. Connelly in this laboratory detected SP-B mRNA on 26 days gestation by Northern

Figure 4.11. Standard curve for the solution hybridization assay. Indicated amounts of SP-B cRNA synthesized in vitro were hybridized with 100,000 cpm of [^{35}S]-antisense SP-B cRNA as described in Section 2.7.2. The recovered hybrids were counted and logarithms of the counts per minute (cpm) remaining after subtracting the cpm obtained with liver total RNA as a blank are plotted against the amounts of SP-B sense cRNA added.

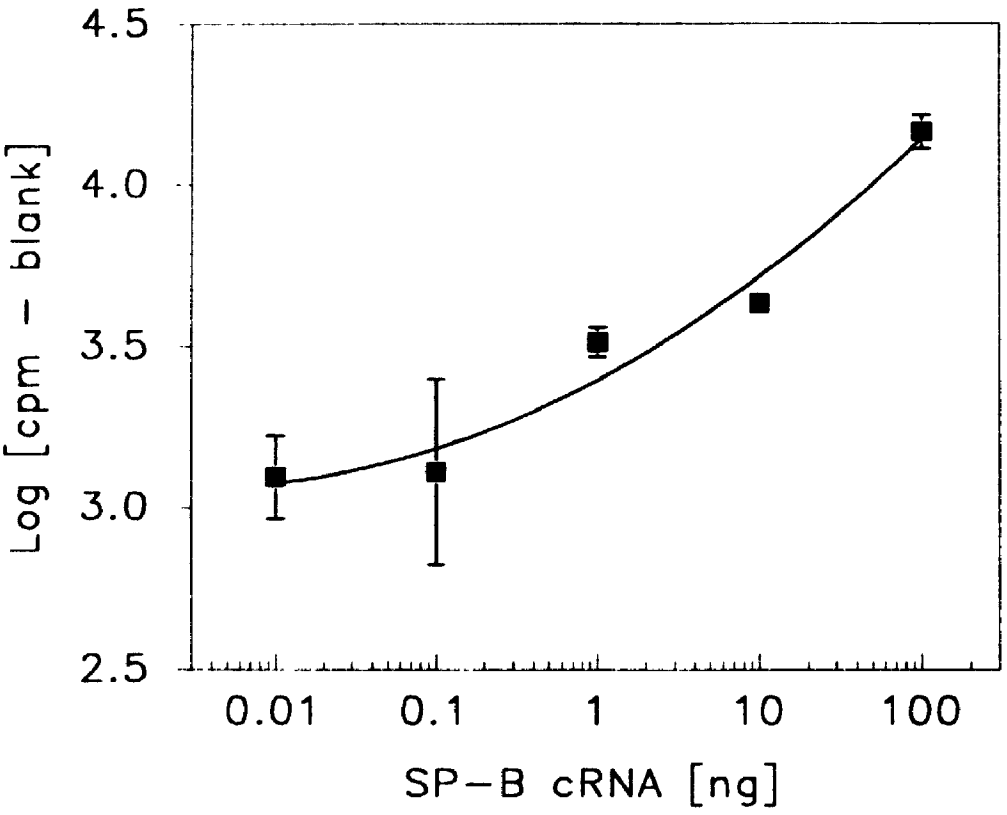
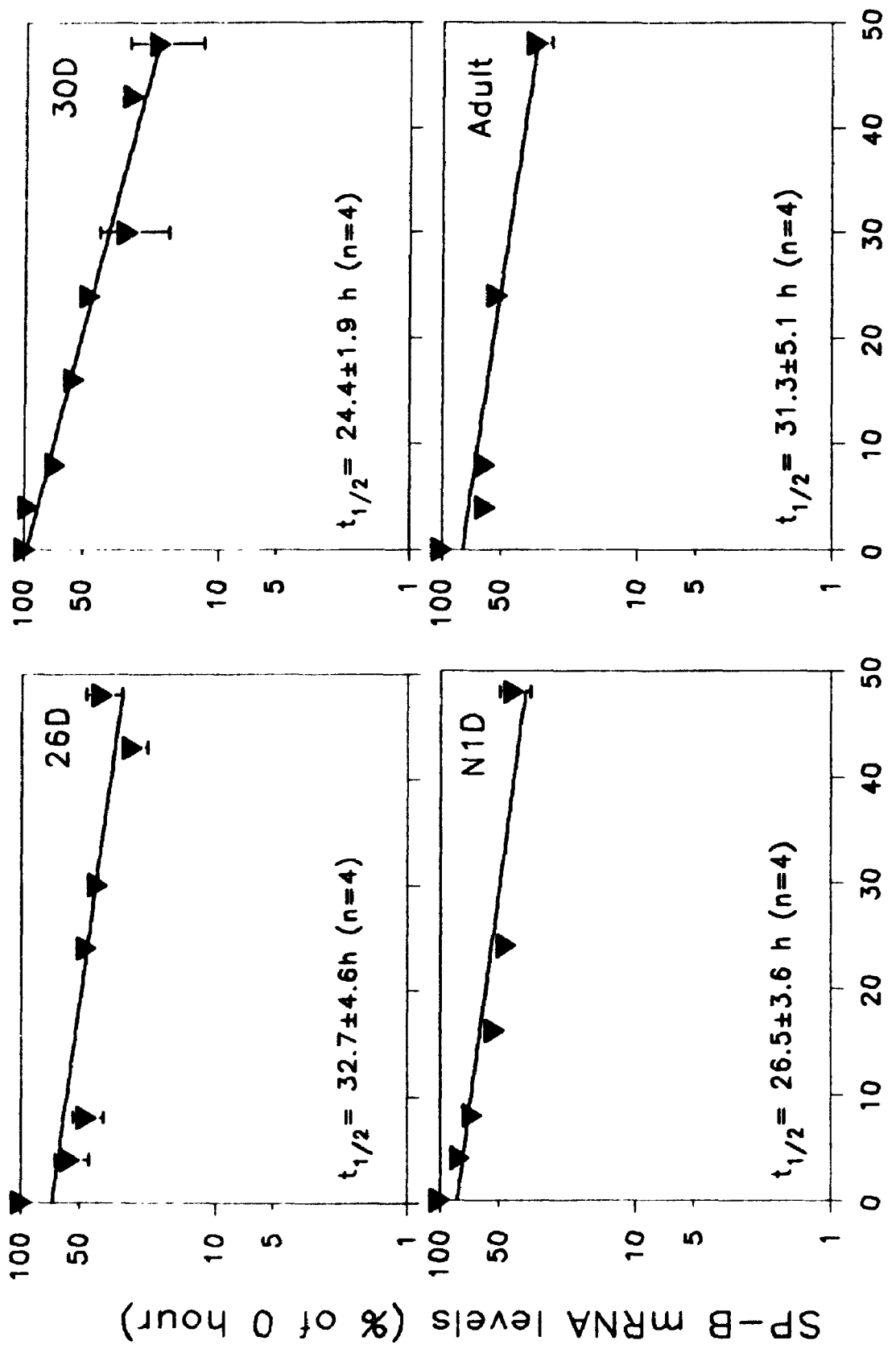

4.5
4.0
3.5
3.0
2.5
Log [cpm - blank]
0.01
0.1
1
10
100
SP-B cRNA [ng]

Figure 4.12. Relative turnover rates of SP-B mRNA in rabbit lung tissue. Explants of 26-days, 30-days gestation, neonatal 1 day and adult rabbit lungs were cultured for up to 48 h in the presence of 1 or 5 μM actinomycin D. Total RNA was isolated from tissues harvested at the times indicated. The pre-existing SP-B mRNA molecules were measured by solution hybridization assay (Fig. 4.7.) and slot blot analysis (Fig. 4.3.). The values are plotted as a percentage of the values at the zero time of addition of the inhibitor. Data are presented as means$\pm$S.E. of four separate experiments for each age. The half-lives of SP-B mRNA at each age calculated from the regression lines are not significantly different from each other.


SP-B mRNA levels (% of 0 hour)
incubation time (h)
26D
$t_{1/2}$= 32.7±4.6h (n=4)
30D
$t_{1/2}$= 24.4±1.9 h (n=4)
N1D
$t_{1/2}$= 26.5±3.6 h (n=4)
Adult
$t_{1/2}$= 31.3±5.1 h (n=4)
100
50
10
5
1
0
10
20
30
40
50

analysis, around the same time that observed presently. However, using a more sensitive technique solution hybridization, he could detect SP-B mRNA as early as 22 days gestation, although at a very low level (Connelly et al., 1991). This discrepancy in the time SP-B mRNA is first apparent in fetal lung may be due to technical reasons, e.g. the sensitivity and the nature of the assays and the manner in which the techniques were applied. We observed in the present study by using slot blot hybridization, which is thought to be more sensitive than Northern analysis, SP-B mRNA was detectable on 22 days instead of 27 days gestation as examined by Northern analysis.

We observed that SP-B mRNA was transcribed, as determined by a well characterized nuclear run-on assay, at relatively high levels as early as 22 days gestation. This level of SP-B mRNA synthesis remained constant until 26 days gestation. Thereafter, a rapid increase was observed in parallel with the elevation in SP-B mRNA levels. Both SP-B mRNA steady-state levels and the relative gene transcription rates reached peaks on 30 days gestation. In neonatal 1 day lung, the SP-B mRNA levels declined slightly but the gene transcription rate remained the same.

Nevertheless, a rapid increase in SP-B mRNA was detected between 26 days gestation and term. This occurs at the same time as the rapid increase in surfactant phospholipid accumulation, in preparation for birth (see Section 1.2.2.1). In the rabbit, type II cells start to differentiate on gestational day 26 (see section 1.2.1.2). The time of initiation of SP-B gene expression in fetal lung tissue (day 22 in this study) is earlier than the appearance of identifiable type II cells. Wohlford-Lenane and colleagues (1992b) using in situ hybridization, first localized SP-B mRNA in pre-alveolar epithelial

cells in fetal rabbit lungs on day 24 of gestation. After 28 days gestation, SP-B mRNA was present in both alveolar pre-type II cells and bronchiolar epithelial cells. Therefore, the cells in which the SP-B gene was expressed during the early part of gestation in rabbit lung was most likely the pre-alveolar epithelial cells or pre-type II cells.

The pattern of SP-B mRNA appearance in the rabbit is relatively similar to that in the rat but differs from that in human. In rabbit and rat lung, SP-B mRNA was detected relatively late in gestation (80% of gestation), whereas in human lung, SP-B is detected rather early (30% of gestation). Despite this close temporal similarity of the developmental expression of the SP-B gene with respect to gestational age in rabbit and rat lung versus human lung, SP-B gene expression occurs at approximately the same time for rat and human fetal lungs in terms of the morphologic phase of development. SP-B mRNA appeared at mid-pseudoglandular in the human, late pseudoglandular in the rat, but during the canalicular phase for the rabbit (see also Section 1.2.1.1 and Fig. 1.3).

Augmented accumulation of SP-B mRNA occurs only after 26 day gestation when differentiated type II cells containing lamellar bodies can first be observed (Kikkawa et al., 1971; Snyder & Magliato, 1991a). This is consistent with the suggestion that type II cells are the major site of SP-B expression. The increase in type II cell numbers which occurs around 26-28 days (Kikawa et a'., 1971), however, makes it uncertain as to whether subsequent in vivo changes in SP-B mRNA levels are the results of an increase in SP-B gene expression per cell, or to the increased number of type II cells, or both. The fact that increases in both SP-B mRNA levels (~3.7-fold) and the SP-B gene transcription (~2.7-fold) are higher than that reported for type II cell number

(~1.7-fold) (Wang et al., 1971) suggested that it could be the result of both increases in gene expression per cell and in the number of type II cells in the lung. In fact, this has been suggested for expression of the other hydrophobic surfactant protein SP-C. Studies with transgenic mice carrying chimeric SP-C-CAT cDNA showed that the concentration of SP-C promoter-directed CAT mRNA and the area that contained this mRNA increased during lung development (Glasser et al., 1991). Although we did not further address this question in our studies, we were able to show a parallel relationship between SP-B gene transcription and SP-B mRNA accumulation during the later stages of gestation and also in neonates (1 day). This suggested the possibility that during this period the developmental regulation of SP-B mRNA levels in vivo could occur primarily at the transcriptional level.

A relatively larger increase in SP-B mRNA levels was observed between 26- day and 28-day fetal rabbit lung compared to that in the SP-B gene transcription rates. This could indicate a longer half-life of this mRNA species and prompted us to examine whether the post-transcriptional mechanisms could be involved in regulation of SP-B mRNA levels at this stage. The half-life of SP-B mRNA was therefore measured using an approach employing actinomycin D. With the realization that this approach could not give absolute values for SP-B mRNA half-life, experiments were conducted with lung tissues from four different age rabbits in order to obtain relative turnover rates for SP-B mRNA. Those experiments indicated that SP-B mRNA was degraded at essentially the same rate during lung development. The values ($t_{1/2}$ = ~30 h) indicated that SP-B mRNA was a very stable message, with a $t_{1/2}$ similar to genes including vitellogenin in

avian liver (26 h, Wiskocil et al., 1980), insulin (29 h, Welsh et al., 1985) and actin (22 h, Krowczynska et al., 1985). There is a possibility that the prolonged half-life of SP-B mRNA hypothesized for 26 day lung occurs in vivo as an end-effect of various hormones present in serum, as partly suggested by the results in chapter 5, or some local agents. This mRNA-stabilizing effect of the circulating hormones or paracrine factors is abolished in vitro where the lung tissue was cultured in serum-free medium for up to 48 h to measure the half life of SP-B mRNA. Another approach using pulse-chase labelling of RNA with radioactive uridine is unfortunately unsuitable for measuring the SP-B mRNA turnover in 26 day lung, since the basal level of the message is too low at this age to obtain reliable values. This latter approach would eliminate the effects of actinomycin D on tissue viability, this experiment would also have to be conducted in vitro and thus would not address the effect of changes in hormonal milieu in vivo.

In summary, SP-B mRNA levels in developing rabbit lung have been examined and compared to that of the SP-B gene transcription rates obtained from the same tissues. It was apparent that SP-B mRNA levels at the later stages of lung maturation, i.e., after 26 days gestation, could be controlled primarily at the transcriptional levels. Although similar relative turnover rates of SP-B mRNA were found in rabbit lungs of 26, 30 days gestation and in neonatal and adult lungs, the possible involvement of post-transcriptional mechanisms in regulating SP-B mRNA levels in vivo could not be ruled out.

CHAPTER 5 - GLUCOCORTICOID REGULATION OF SP-B mRNA LEVELS DURING LUNG DEVELOPMENT

5.1. Introduction

The first evidence for glucocorticoid control of fetal lung maturation was reported by Liggins (1969) who noted, quite by accident, that antenatal administration of glucocorticoids increased survival of prematurely delivered (120/147 days gestation) lambs. Since that time glucocorticoids have received considerable attention and it has been demonstrated that glucocorticoids accelerate many aspects of lung maturation including morphogenesis, type II cell differentiation and consequently pulmonary surfactant synthesis. As a result, several clinical trials have been undertaken which have clearly demonstrated that antenatal glucocorticoid administration can reduce the incidence of respiratory distress syndrome (for review see Ballard et al., 1992).

There is abundant evidence that glucocorticoid treatment increases the amount of surfactant lipids in fetal lung lavage, stimulates formation of phosphatidylcholine from labelled choline in lung slices, and decreases the glycogen content of fetal lung. The activities of several enzymes involved in surfactant phospholipid synthesis have been reported to be increased in whole-lung tissue by glucocorticoid adminstration in vivo (for extensive review see Post and van Golde, 1988; Robertson et al., 1992).

Considering the importance of surfactant proteins for surfactant function, it was of interest to investigate the glucocorticoid regulation of surfactant protein gene expression during lung development. At the time we started this study, SP-A was under

extensive investigation while SP-B had just began to attract attention. Glucocorticoids, such as dexamethasone or cortisol, are able to increase SP-B mRNA accumulation in human fetal lungs of 15-20 weeks gestational age, in lung explant systems (Whitsett et al., 1987; Liley et al., 1989) and in rat or rabbit lung in whole animals (Schellhase and Shannon, 1991; Connelly et al., 1991; Fisher et al., 1991). Shimizu and co-workers observed that maternal glucocorticoid treatment also increased SP-B content in fetal rat lungs (Shimizu et al., 1991). In human lung explants, the increase in SP-B mRNA levels appears to be associated with an increase in SP-B protein level, however it should be noted that this increase was detected by an antiserum raised against a combination of both SP-B and SP-C (Whitsett et al., 1987). Dose-dependent increases in both SP-B mRNA and protein content were reported by O'Reilly et al. in a study in which a H-820 adenocarcinoma cell line was incubated with various concentrations of dexamethasone (O'Reilly et al., 1991). It has been observed that glucocorticoids can increase SP-B gene transcription in a lung tumour cell line (O'Reilly et al., 1991). However, the effects of this hormone on transcriptional activity of the SP-B gene have not been studied in developing lung.

In this chapter, we studied the control of SP-B mRNA levels by glucocorticoids and the possible mechanisms which might be involved. We used the rabbit fetus as a model because of our previous experience with surfactant lipid synthesis and because the rabbit has a relatively long gestational period (31 days) which allows examination of many more stages of lung development than would be possible using the rat or mouse fetus as a model. The effect of the synthetic glucocorticoid, dexamethasone, on SP-B

mRNA accumulation as well as SP-B gene transcription was examined in fetal rabbit lung of two different ages which were 26 day gestation, when differentiated type II cells start to appear, and 30 day gestation, one day before term. The mechanisms by which dexamethasone exerts its effects were also studied. The response of SP-B protein synthesis to dexamethasone treatment was only briefly examined, for lack of a suitable antibody.

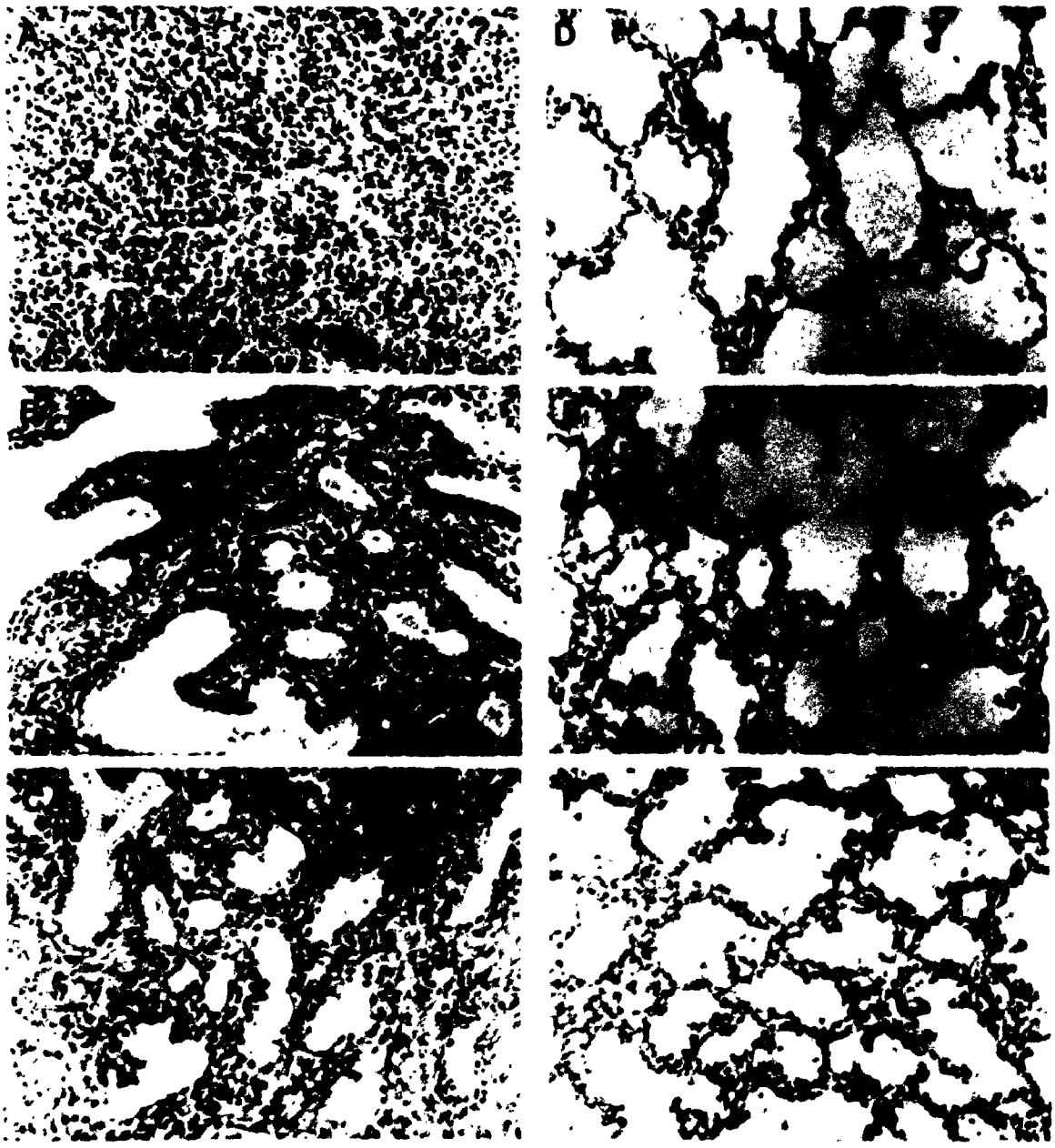
5.2. Results

5.2.1. Fetal lung in organ culture

Lung explant organ culture has been used successfully to study morphological and biochemical maturation of fetal lung as regulated by various hormones (Snyder et al., 1981; Gross & Wilson, 1982; Whitsett et al., 1987c). The system has been well established and characterized. We adapted previous methods by combining aspects of the procedures published by Snyder et al. (1981) and Whitsett et al. (1987c) as follows: (1) lung tissue diced into 1 mm^3 cube (see Section 2.2) were incubated at 37°C in defined serum-free Waymouth medium on the top of a nylon membrane which was supported by a stainless-steel grid; (2) the medium was added to a level where the top of the tissue was exposed directly to 95% air and 5% CO_2; and (3) the culture was rocked from side to side at a very low speed during incubation.

A portion of the culture tissue was fixed in 10% formalin followed by routine light microscopy. As seen in Fig. 5.1 (A), preculture lung tissue of day 26 gestational age rabbit was in the pseudoglandular or early canalicular stage of development. Within

Figure 5.1. Light microscopic histology of 26-and 30-day fetal rabbit lung tissue after various times in explant culture. The sections were stained with hemotoxin and eosin (45X). **(A)** 26-day fetal rabbit lung (preculture). **(B)** Explants of 26-day fetal lung maintained for 3 days in control medium. **(C)** Explants of 26-day fetal lung maintained for 1 day in control medium and 2 days in medium containing 10^{-8}M dexamethasone. **(D)** 30-day fetal rabbit lung (preculture). **(E)** Explants of 30-day fetal lung maintained for 3 days in control medium. **(F)** Explants of 30-day fetal lung maintained for 1 day in control medium and 2 days in medium containing 10^{-8}M dexamethasone.

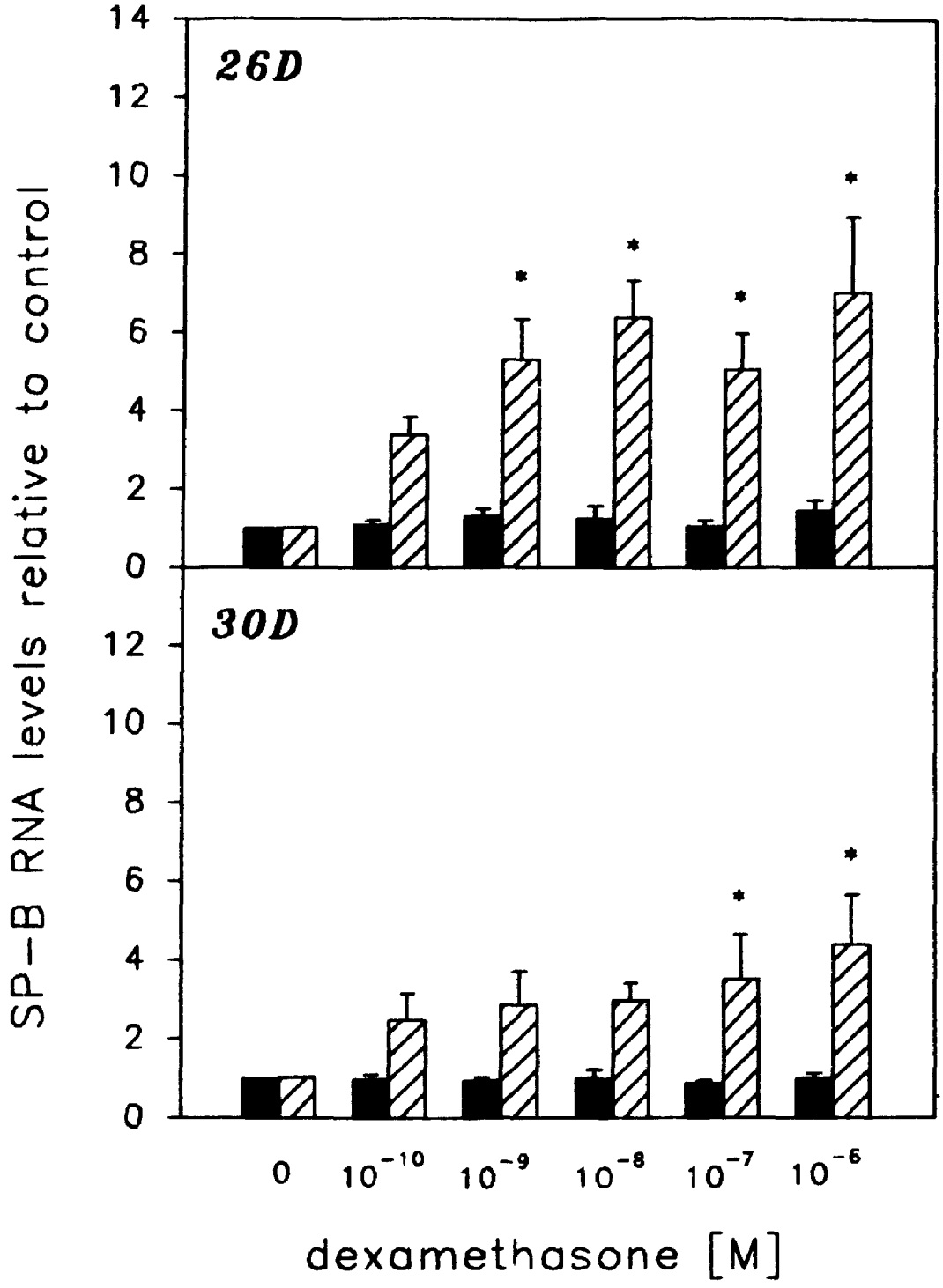

A
B

48 h in organ culture, the lumen of the ducts enlarged (Fig. 5.1. **B**). The epithelium and connective tissue of lung maintained in the absence of dexamethasone appeared viable and basically intact. The addition of dexamethasone at 10^{-8}M to the media had no discernible effect on tissue morphology when compared to control explants (Fig.5.1. **C**). The preculture lung tissue of 30 days gestation rabbit, shown in Fig. 5.1. **D**, was in the saccular stage of development which appears as large irregularly-formed lumina with thin walls composed of both cuboidal and flatten cells. After 48 h explant culture, with (Fig. 5.1. **F**) or without (Fig. 5.1. **E**) dexamethasone, the luminal walls became thicker. Some cells were observed within the alveolar spaces. Extensive cell death was not observed in any of the conditions studied.

5.2.2. Effect of various concentrations of dexamethasone on the levels of SP-B mRNA and on SP-B gene transcription

The parallel relationship observed in the developmental profiles of SP-B mRNA and SP-B gene transcription (Figs. 4.1 and 4.2) implies that the levels of SP-B mRNA in fetal lung could be regulated primarily at the transcriptional level. As stated earlier, glucocorticoids can increase SP-B mRNA levels in lung tissue (for review see Weaver and Whitsett, 1991; Mendelson & Boggaram, 1991). To determine the level at which glucocorticoids are responsible for this alteration and whether there is a difference between the response of lung tissues at early and late stages of lung maturation, explants of 26-day and 30-day fetal rabbit lungs were cultured for 72 h with addition of dexamethasone at concentrations of 10^{-10}-10^{-6} M after 24 h of culture (i.e., 48 h exposure

Figure 5.2. Dexamethasone dose response curve for SP-B gene transcription and mRNA levels. Explants of 26-day and 30-day fetal rabbit lung were cultured for 3 days with dexamethasone at various concentrations being present during the last 2 days of culture. Transcriptional activities of the SP-B gene were assessed by nuclear run-on analysis and SP-B mRNA levels were analyzed by slot blot analysis. Autoradiograms obtained from the two assays were subjected to densitometry. Transcriptional activity *(solid bars)* of the SP-B gene and SP-B mRNA levels *(hatched bars)* are presented as values relative to control (=1). Data are the means±SE for three separate experiments. Treatment with dexamethasone at 10^{-9} to 10^{-6}M on days 26 and 10^{-7} to 10^{-6}M on 30-days explants significantly increased SP-B mRNA levels compared to those of the control ($P < 0.05$) but showed no effect on transcription of the SP-B gene.


26D
30D
SP-B RNA levels relative to control
0
2
4
6
8
10
12
14
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
dexamethasone [M]

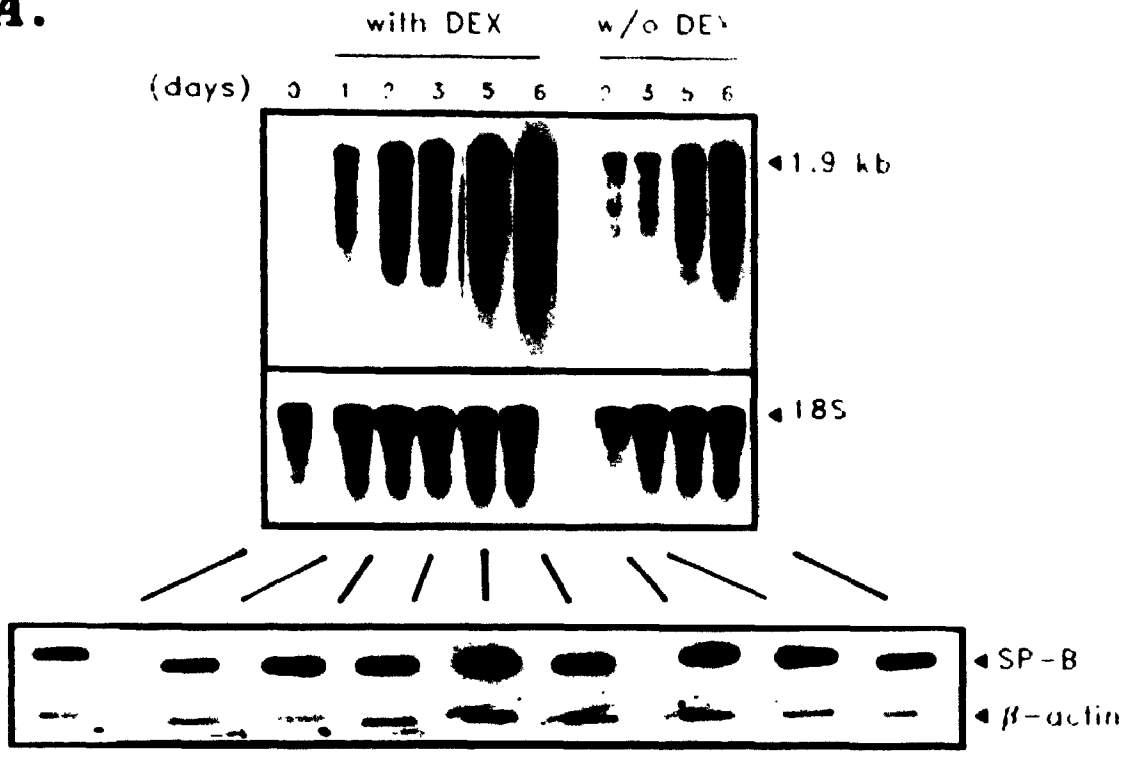
to hormone). Exposure of lung explants from fetuses of 26-days gestation to dexamethasone at 10^{-9}-10^{-6} M led to a 6-fold increase ($P<0.05$) in SP-B mRNA levels relative to tissue incubated in the absence of steroid (Figs.5.2). With explants of 30 days gestation, dexamethasone concentrations of 10^{-7} M and 10^{-6} M led to a modest, but still significant, elevation in SP-B mRNA levels. Interestingly, at the concentrations used in this study, dexamethasone did not have any significant effect on SP-B gene transcription at either gestational age.

5.2.3. Effects of explant culture and dexamethasone treatment on SP-B gene transcription and the levels of SP-B mRNA as a function of incubatio.. time

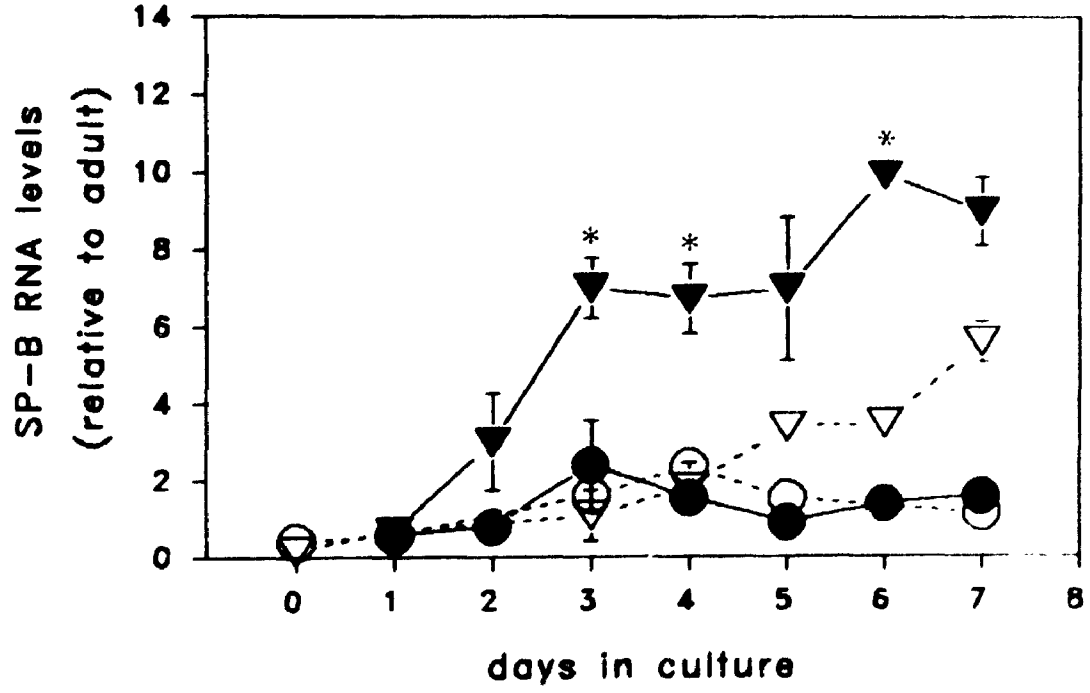
The effect of dexamethasone on the levels of SP-B mRNA was further investigated by culturing explants for up to 7 days with hormone at 10^{-8} M added after first 24 h. With explants from fetuses of 26-days gestation cultured in the absence of dexamethasone (Fig. 5.3), SP-B mRNA levels exhibited a gradual increase which accelerated between day 4 and 7 resulting in levels approximately 5-fold ($P<0.05$) greater than those observed with adult tissue. Dexamethasone at 10^{-8} M produced a rapid increase in SP-B mRNA steady-state levels to 7-fold adult levels within 48 h of treatment. By days 6 and 7, SP-B mRNA levels were 8- to 10-fold greater than in adult lung. The level of SP-B mRNA initially in fetal lung at 30-days gestation was somewhat greater than that observed in adult lung (Point 0 in Fig.5.4). Incubation in the absence of dexamethasone led to a very gradual increase in mRNA to levels 3.5 times that observed in the adult. Addition of the steroid at 10^{-8} M resulted in a moderate increase

Figure 5.3. Day-26 lung SP-B mRNA and nuclear run-on transcript levels in response to dexamethasone treatment. Fetal rabbit lung explants were cultured without hormone *(dash lines, open symbols)* or with 10^{-8} M dexamethasone *(solid lines, closed symbols)* added on day 1. *A*, representative Northern blot (probed with SP-B, 1.9 kb, and 18s rDNA) and nuclear run-on (SP-B and β-actin) analyses. Nuclei were isolated from the tissues before (point 0) and after the indicated times of dexamethasone exposure. SP-B gene transcription *(lower panel or circles in B.)* was analyzed by transcription run-on analysis; SP-B mRNA levels *(triangles in B)* were assessed by Northern *(upper panel)* and slot blot analysis. *B*, autoradiograms from run-on assays and slot blot hybridization assays were subjected to densitometry and SP-B gene transcription and mRNA levels are plotted relative to the respective adult values. The data are present as the means$\pm$SEM for three separate experiments. * indicates values are significantly different from the corresponding control values ($P<0.05$).

A.


with DEX
w/o DEX
(days) 0 1 2 3 5 6 2 3 5 6
1.9 kb
18S
SP-B
β-actin

B.


SP-B RNA levels
(relative to adult)
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8
days in culture
*
*
*

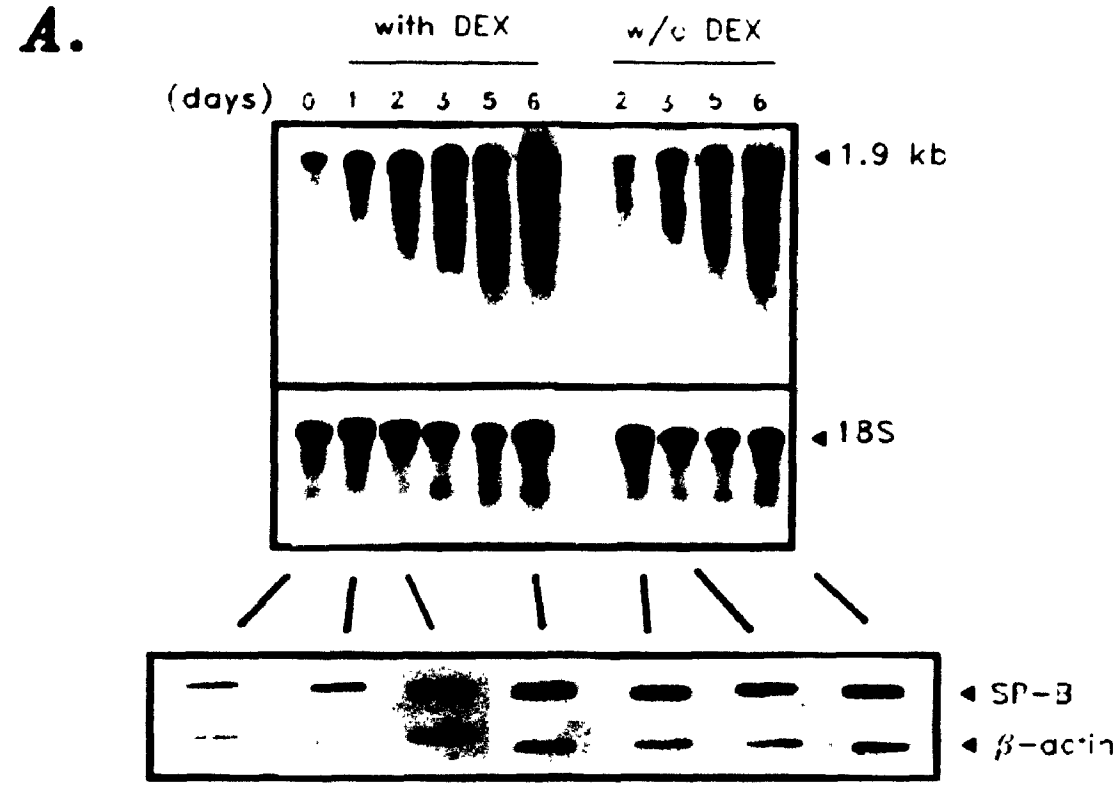
in mRNA to levels 3-fold greater than adult at 48 h and 6-fold greater than adult at 72 h of treatment. This response was similar to, but more moderate than, that observed with explants of 26-days gestation (compare Fig. 5.4 and Fig. 5.5).

Initially, transcription rates of the SP-B gene were low in fetal lung of 26-days gestation (Fig. 5.4) but increased to levels approximately twice that in the adult by day 4 in culture with control medium. The exact same pattern was observed when dexamethasone at 10^{-8} M was present. With explants of 30-days gestation (Fig. 5.5), the transcription rates fell during the first day in culture from a level similar to that in adult lung to low values. Thereafter transcription rates increased over the next 48 h to levels close to those observed with nuclei from adult lung. As with 26 day explants, dexamethasone at 10^{-8} M did not cause any further increase in the transcription rates of the SP-B gene.

Treatment of fetal lung explants with dexamethasone had no significant effect on either the overall transcriptional activity of the nuclei or on the amount of total RNA isolated per gram weight of tissue. Except for a small decrease during the first day with 30 day lung, the protein/DNA ratio did not change during lung explant culture (Fig. 5.5). No differences in overall transcription activity and protein/DNA ratios were observed between the day 26 and day 30 explants during culture (data not shown).

The suggestion that an increase in SP-B mRNA accumulation upon dexamethasone treatment was mainly due to post-transcriptional mechanisms was further supported by data from experiments in which lung explants were cultured in presence of dexamethasone and actinomycin D for 48 h after 24 h in control medium. The mRNA

Figure 5.4. Day-30 lung SP-B mRNA and nuclear run on transcript levels in response to dexamethasone treatment. The experiment was conducted as for Fig. 5.3. except fetal lungs of 30-days gestation were used. The symbols are assigned as in Fig. 5.3.


A.
with DEX
w/o DEX
(days) 0 1 2 3 5 6 2 3 5 6
1.9 kb
18S
SP-B
β-actin

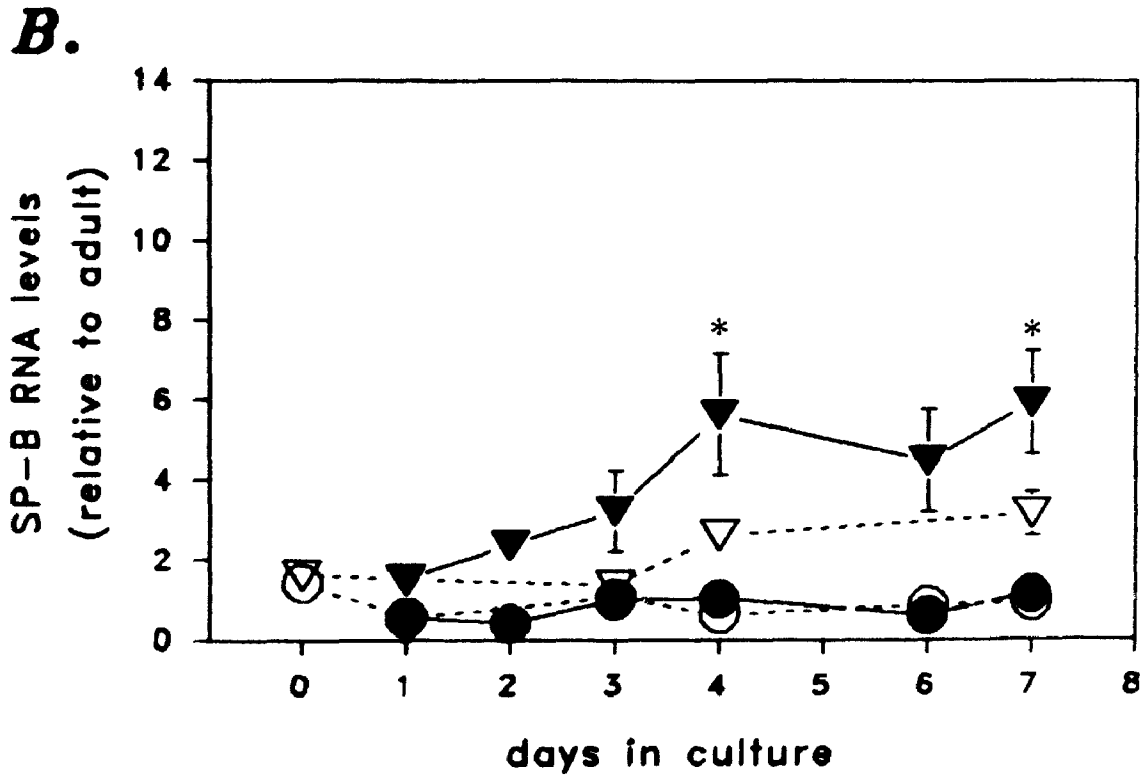
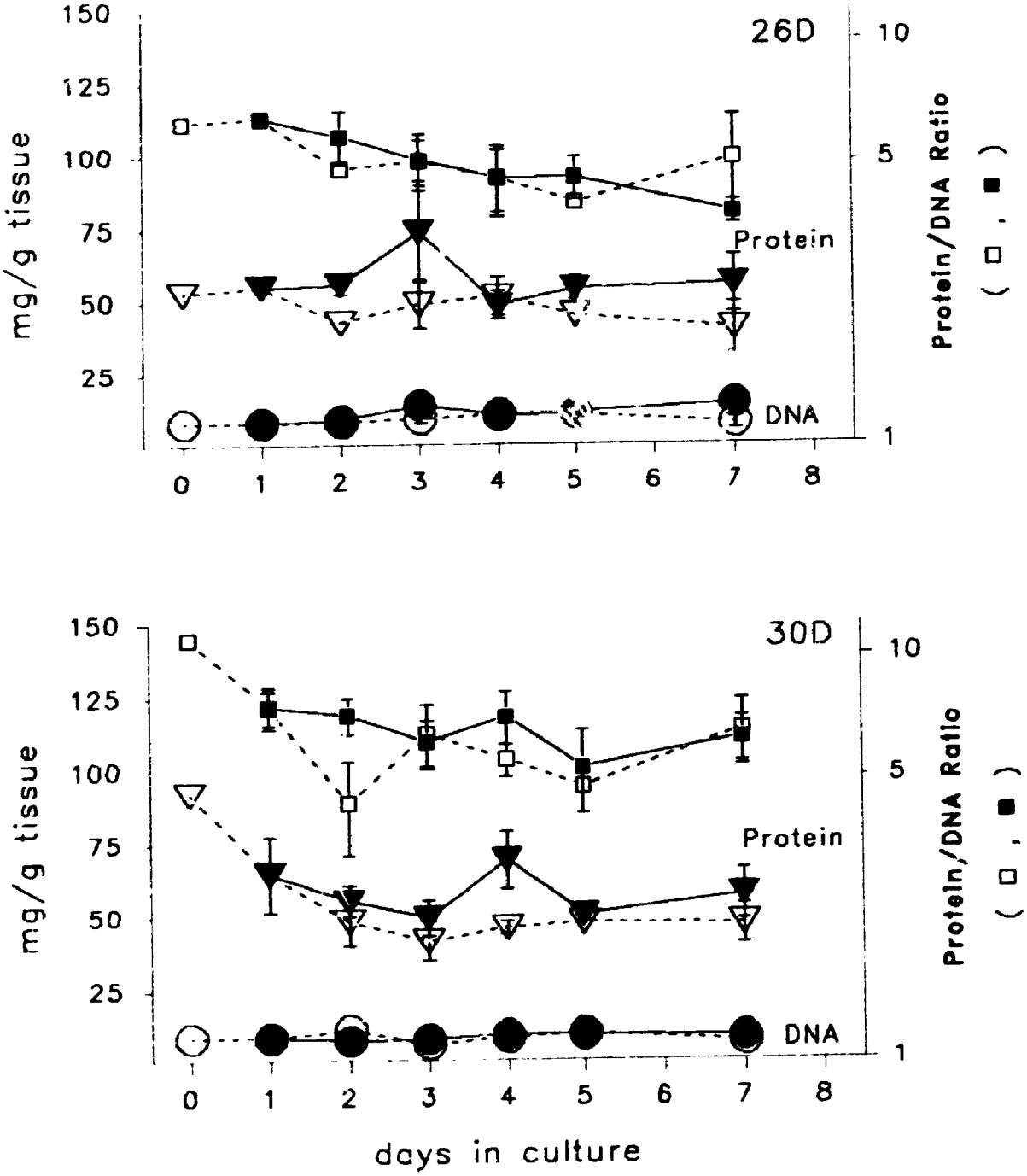

B.
SP-B RNA levels
(relative to adult)
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8
days in culture
*
*

Figure 5.5. DNA, protein contents and DNA/protein ratios of 26-day and 30-day fetal lung tissues during explant cultures (Fig. 5.3 and 5.4). DNA and protein contents of tissue homogenates were determined using standard methods. Statistical analyses showed no significant differences in DNA *(circles)* or protein *(triangles)* contents or in DNA/protein ratios *(squares)* during lung explant cultures of gestational day-26 (26D) and day-30 (30D) fetuses in the presence *(closed symbols)* or absence *(open symbols)* of dexamethasone.


26D
30D
Protein
DNA
mg/g tissue
Protein/DNA Ratio
(□ , ■)
150
125
100
75
50
25
10
5
1
0
1
2
3
4
5
6
7
8
days in culture

levels were decreased to a very low level by treatment with actinomycin D alone. Addition of dexamethasone retained the SP-B mRNA at levels similar to that of control (Fig. 5.6). This suggested that dexamethasone-induced increase in SP-B mRNA could be mediated by stabilizing the SP-B message.

5.2.4. The effect of dexamethasone on SP-B mRNA accumulation requires ongoing protein synthesis

When day 26 or day 30 gestation fetal lung was cultured under the same conditions as in Section 5.2.3. but with the addition of 10 μM cycloheximide, the SP-B mRNA steady-state levels were reduced to less than half of the control (48 h in control medium) value (Fig.5.7). The transcription of the SP-B gene as measured by nuclear run-on assays was not affected by the inhibitor in lung explants of either age. This suggests that ongoing protein synthesis is necessary for the dexamethasone-induced increase in SP-B mRNA levels and that post-transcriptional mechanisms are most likely involved since no net response to dexamethasone treatment was detected at the transcriptional levels.

When lung explants of both 26 and 30 day fetuses were incubated first in control medium for 24 h then with 10 μM of cycloheximide. the SP-B mRNA levels were decreased to about half of that incubated in control medium for 48 h. Interestingly, SP-B gene transcription increased in 26 day explants to ~ 1.5-fold over the control value while it decreased in 30 day explants as much as in the control levels (Fig. 5.7). This was not observed with SP-A, SP-C or 18s gene transcription (Fig. 6.7 and 6.8). This result,

Figure 5.6. Effect of dexamethasone on SP-B mRNA levels in the presence and absence of actinomycin D. Gestational age 30-day rabbit lung explants were cultured with 10^{-8} M dexamethasone added on day 1. The culture was continued for a further 48 hours in the presence (DEX+ActD) or absence (DEX) of 5 μM actinomycin D. Explants with the inhibitor added alone on day 1 (ActD) were also examined. SP-B mRNA levels were determined by slot blot hybridization of total RNA isolated from the explant tissues. Data obtained upon densitometry are presented as values relative to control values (means$\pm$S.E., n=3).

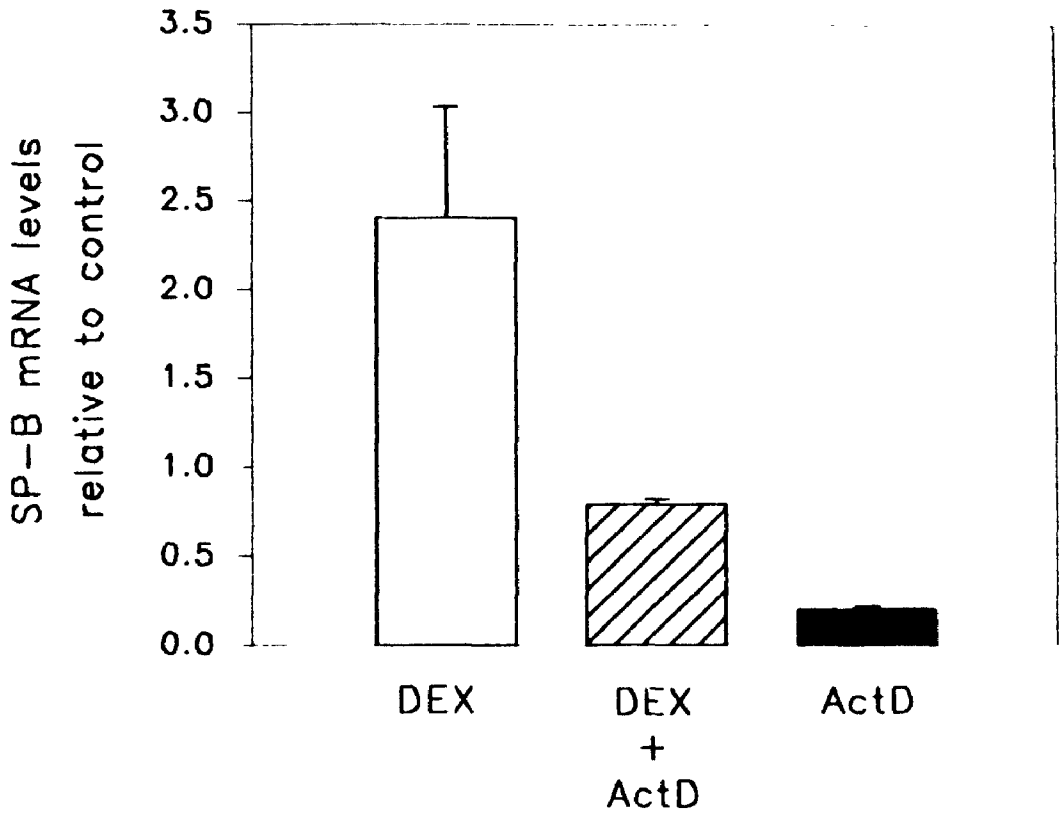
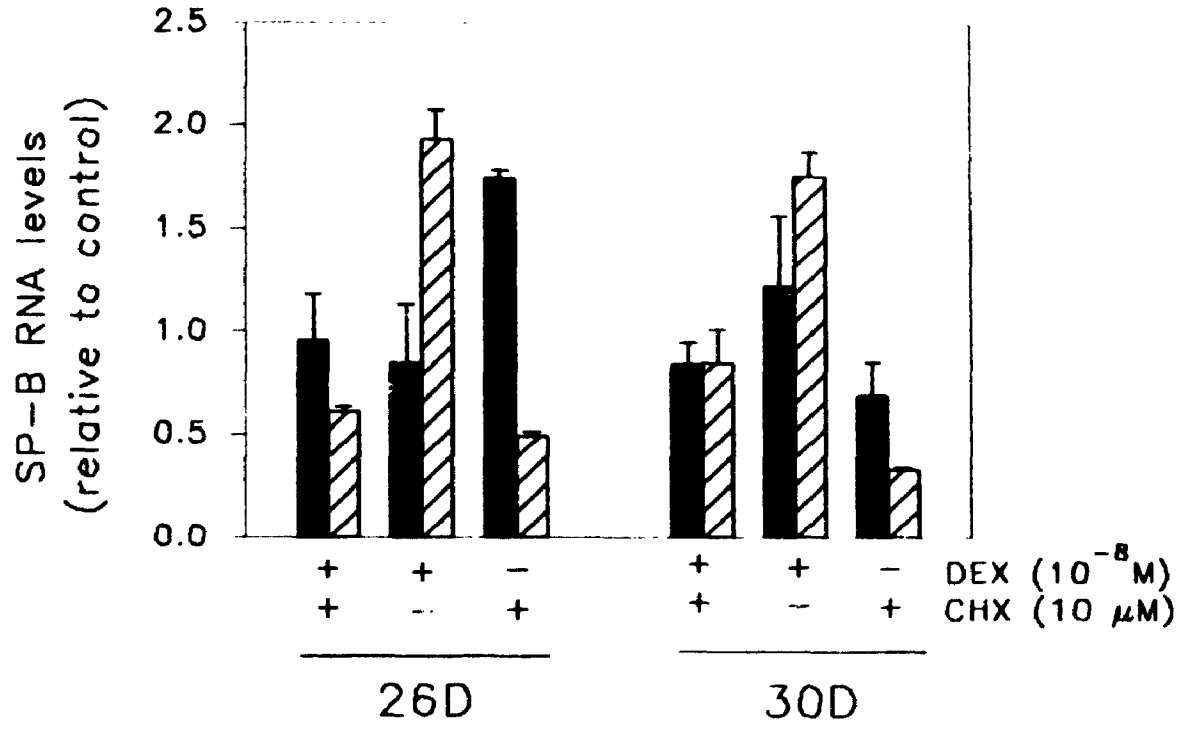

SP-B mRNA levels
relative to control
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
DEX
DEX
+
ActD
ActD

Figure 5.7. Effect of dexamethasone on SP-B mRNA levels in the presence or absence of cycloheximide. Lung explants of 26- and 30-day gestations were maintained in culture for 24 h. Either dexamethasone (10^{-8} M), cycloheximide (10 μM) or the two agents in combination were added and the explants cultured for a further 24 h. Nuclei and cytoplasmic RNA were isolated and subjected to nuclear run-on assays or slot blot hybridization, respectively, using rabbit SP-B cDNA as a probe to measure SP-B gene transcription *(solid bars)* and the message level *(hatched bars)*. Densitometric data of the autoradiograms (see Fig. 6.7.) are presented as values relative to that of explants maintained in control medium (means$\pm$S.E., n=3).


SP-B RNA levels
(relative to control)
2.5
2.0
1.5
1.0
0.5
0.0
+ + − + + −
+ − + + − +
DEX (10^{-8}M)
CHX (10 μM)
26D
30D

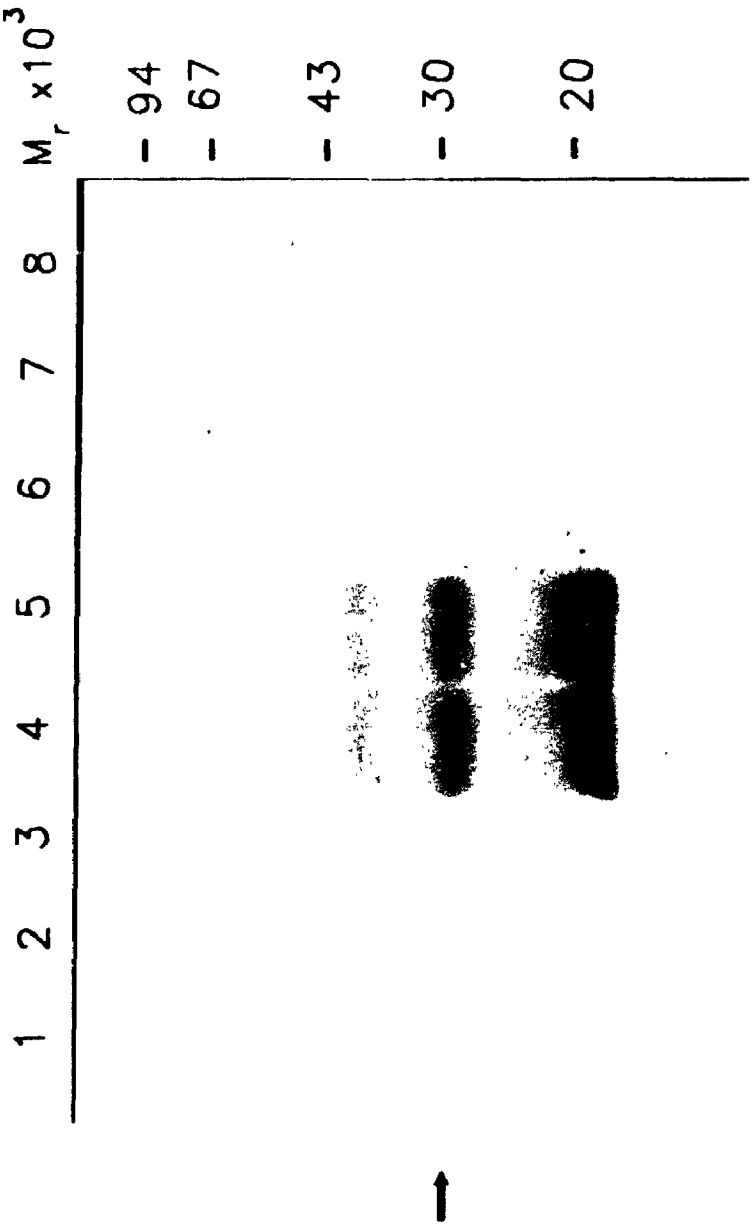
however, needs to be confirmed with some other protein synthesis inhibitors such as puromycin.

5.2.5. Examination of the effect of in vitro dexamethasone treatment on the appearance of SP-B protein

Examination of the effect of glucocorticoid hormone on prenatal appearance of surfactant protein-B was also attempted. Preculture, control and dexamethasone-treated explant tissues of fetal lungs on day 26 and day 30 of gestation were studied. A mouse monoclonal SP-B antibody (#1B9, obtained from Dr. Whitsett) was used to detect SP-B protein from lungs homogenized in 1x PBS/1 mM PMSF (Shimizu et al., 1991). This antibody was raised against mature human SP-B and is able to detect the natural dimeric form of SP-B from human, bovine and sheep lung surfactant. The results of Western blot analysis revealed a protein with molecular weights of approx. 40,000 in the absence of reducing reagent (Fig. 5.8) and approx. 38,000 under reducing condition (data not shown). The content of the peptide did not appear to change with different culture conditions. No proteins of 8,000 - 18,000 molecular mass, which correspond to reduced and non-reduced mature SP-B in other species, were detected. Whether or not this high molecular weight protein is rabbit SP-B could only be confirmed using a second heterologously generated antibody, which unfortunately is not available at the present time.

5.3. Discussion

Figure 5.8. Immunoblot analysis of SP-B in homogenates of fetal lung tissue. Forty micrograms of protein from lung homogenate of fetal rabbits of 26- and 30-days gestational age before or after being cultured for 48 h in the presence or absence of 10^{-8}M dexamethasone were separated by one-dimensional SDS-PAGE (12%, non-reducing conditions), then analyzed for SP-B protein by an immunoblotting technique (section 2.12.2) using monoclonal antibody (1B9) directed against mature human SP-B. The arrow indicates the position of a protein which reacts with the antibody. The samples for each lane were: lane 1 - homogenate of 26-day lung, preculture; lane 2 - homogenate of 26-day lung explants in control medium for 48 h; lane 3 - homogenate of 26-day lung explants in medium containing dexamethasone; lanes 4 & 5 - purified bovine mature SP-B proteins; lane 6 - homogenate of 30-day lung, preculture; lane 7 - homogenate of 30-day lung explants in control medium for 48 h; lane 8 - homogenate of 30-day lung explants in medium containing dexamethasone for 48 h.


$M_r \times 10^3$
94
67
43
30
20
1
2
3
4
5
6
7
8

Numerous studies have shown that glucocorticoids can accelerate the morphological, physiological and biochemical maturation of fetal lung in vivo and in vitro (for review see Snyder, 1991; Ballard, 1989; Gross, 1990, Weaver and Whitsett, 1991; Mendelson and Boggaram, 1991; Post, 1992). In the present study, it was attempted to determine the level at which glucocorticoids affect SP-B mRNA accumulation during development. The approach used was to examine the steady-state levels of SP-B mRNA as well as transcription of the SP-B gene in explant cultures of fetal rabbit lungs of 26- and 30-day gestation, representing early and late stages of lung maturation respectively. In agreement with previous studies conducted with rat (Floros et al., 1991) and human (Whitsett et al., 1987c; Liley et al., 1989) lung, we observed that dexamethasone increased SP-B mRNA accumulation in rabbit fetal lung explants in a dose- and time-dependent manner. The stimulatory effect of dexamethasone on SP-B mRNA levels at 30-days gestation, however, was slower and attained a relatively lower final level than that observed at 26-days (6-fold vs 8-10-fold of the adult levels) despite the fact that with 30-day lung, the initial mRNA levels were much higher. Using transcriptional run-on assays, we were not able to observe any effect of dexamethasone on the relative rates of transcription of the SP-B gene in lung explants of either gestational age during the time periods examined. The findings presented here regarding control of SP-B gene expression differ considerably with observations on SP-A gene regulation where dexamethasone exerts dose-dependent effect on both SP-A mRNA accumulation and its relative gene transcription rates (Boggaram et al., 1989) in that increasing concentrations of dexamethasone from 10^{-10}M to 10^{-7}M caused an increase in

the SP-A gene transcription but a decrease in SP-A mRNA levels.

Thus, dexamethasone treatment of rabbit fetal lung led to a rapid increase in SP-B mRNA levels but had no significant effect on the transcription rate of the gene. These findings are consistent with the observation by O'Reilly et al. (1991) that, in a human pulmonary adenocarcinoma cell line (H441), dexamethasone caused 60- to 150-fold increases in SP-B mRNA levels but only a 2- to 4-fold elevation in SP-B gene transcription. These findings suggest that the major effects of dexamethasone on SP-B mRNA levels in vitro are at the post-transcriptional level. This is supported by the present observation that dexamethasone was able to retain SP-B mRNA detected in lung explants cultured with actinomycin D at a level near that of the control whereas the inhibitor alone reduced the message to a very low level. The mechanism by which glucocorticoids cause SP-B mRNA retention in lung tissue is not well established. It has been hypothesized that the hormone could prolong the half-life of SP-B mRNA. Venkatesh and co-workers recently reported that, by using approaches such as application of actinomycin D and pulse-chase labelling, SP-B mRNA half-life was found to be increased by dexamethasone from 6 h to 16 h in human fetal lung explants (Venkatesh et al., 1993). This increase in SP-B half-life could be mediated by a mRNA stabilizing factor induced by the presence of the hormone. As noted earlier in chapter 3, several AU-rich regions which resemble the AUUUA-motifs have been found in the 3' untranslated regions of the rabbit and human SP-B mRNA sequences. Therefore, AU-binding proteins or other factor(s) induced by the hormone could be involved in this message-stabilizing process. The fact that the increase in SP-B mRNA in response to

dexamethasone can be blocked by protein synthesis inhibitors (Fig. 5.8 and O'Reilly et al., 1991) further supports the suggested existence of a labile protein(s). This latter observation, however, is not consistent with that reported by Venkatesh et al. (1993). These researchers observed that incubation with cycloheximide for 8 h did not alter SP-B mRNA levels in human fetal lung explants. The reasons for these different observations are not clear. It could be due to the different length of time cycloheximide was present in the culture used in their studies (8 h) and studies present here (24 h) and reported by O'Rilly et al. (1991). It has been noticed that an exposure time of 24 h was needed to observe a significant decrease in surfactant phospholipid synthesis in human fetal lung after treatment with the inhibitor (Gross et al., 1983). Nevertheless, the observation that dexamethasone caused a significant elevation in SP-B mRNA content but not gene transcriptional activity indicates that glucocorticoids alone cannot be fully responsible for the increase in SP-B gene transcription which is observed during lung development in vivo (Chapter 4). The possibility that glucocorticoids may act in concert with other hormones and factors to enhance SP-B gene transcription must still be considered.

The abundance of SP-B mRNA in 26-day (84% gestation, Fig. 1.5) rabbit lung explants increased steadily during the first 4-6 days in control medium and then rose more rapidly to levels 4-6 fold over adult levels. Day 26 of gestation marks the transition from pseudoglandular to canalicular stage of rabbit lung development (see Fig. 1.5., also Wang et al., 1971; Kikkawa et al., 1971; Snyder et al., 1991a; Possmayer et al., 1984). With lung cultures from 30 day fetuses (96% gestation, canalicular stage), SP-B mRNA levels were initially above adult levels and increased slowly to levels

approximately 3-4 fold greater than in the adult. SP-B mRNA levels also increase spontaneously when human lung of 15-18 weeks gestation (~40% gestation) are incubated in organ culture (Liley et al., 1989). It has been reported that the spontaneous maturation of lung explants is related to increased tissue cAMP levels, arising from endogenous prostaglandin production (Ballard et al., 1990; Mendelson et al., 1986). The cAMP analog, Bt_2cAMP, markedly stimulates SP-A gene transcription (Boggaram et al., 1989) and the accumulation of SP-A mRNA and protein (Odom et al., 1987, 1988; Boggaram et al., 1991). Likewise, forskalin (increasing endogenous cAMP) and terbutaline (a β-adrenergic receptor agonist) as well as Bt_2cAMP increased SP-B mRNA levels in human lung explants but to a lesser extent than dexamethasone (Liley et al., 1989; Whitsett et al., 1987a and 1987b). Whether cAMP or prostaglandins can increase SP-B gene transcription has not been reported.

Treatment of pregnant does at 26-days gestation with betamethasone led to a doubling of SP-B mRNA levels in fetal lung within 24 h (Connelly et al., 1991). Dexamethasone treatment of pregnant rats in early gestation (17-19 days, term 22 days) led to a greater increase in relative SP-B mRNA levels in fetal lung than at late gestation or in neonates but this was clearly related to the lower control levels (Schellhase and Shannon, 1991). Daily administration of dexamethasone to pregnant rats for 3 days resulted in a larger response in fetal SP-B mRNA on day 19 than a single dose on either day 17 or 19 of gestation. Similar SP-B mRNA levels were observed on day 19 of gestation after either 3 or 5 days of dexamethasone treatment. These results show that glucocorticoids can influence fetal SP-B mRNA levels in vivo as well as in vitro.

Although the mechanism in vivo is not necessarily the same as in vitro, it is interesting to note that, as reported in the present study, glucocorticoid treatment in early gestation led to a greater relative, but not necessarily absolute, effect on fetal lung SP-B mRNA (Phelps and Floros, 1991; Schellhase and Shannon, 1991) and that the maximal effect was observed by 72 h of exposure (Schellhase and Shannon, 1991).

The increase in SP-B gene transcription observed in the present study in nuclei isolated from 26-day rabbit fetal lung explants cultured with cycloheximide (10 μM) alone needs to be further confirmed. This increase could reflect a counteraction of an inhibitory protein which attenuates SP-B gene transcription in lungs at this gestational age. It could also reflect a blockage of degradation of the newly synthesized hnRNA during the process of nuclear run-on assay, however only a slightly decrease in [^{32}P]SP-B transcript levels was evident during the characterization of the assay (see Fig. 4.5). Further experiments are needed to clarify this matter.

In summary, it was clearly demonstrated in this study that in vitro dexamethasone treatment had a stimulatory effect on SP-B mRNA levels whileas no detectable effect on SP-B gene transcriptional activity. This effect of dexamethasone on SP-B mRNA accumulation was most likely due to a post-transcriptional mechanism. This was supported by the fact that SP-B mRNA levels were retained by the hormone to a level close to control in lung explants incubated with dexamethasone and actinomycin D, whereas actinomycin D alone caused a marked reduction in the message levels. This messenger-elevating effect of the hormone was blocked by a protein synthesis inhibitor while transcription rate of the SP-B gene was not altered.

CHAPTER 6 - ONTOGENY AND GLUCOCORTICOID REGULATION OF SP-A AND SP-C mRNA LEVELS IN RABBIT LUNG

6.1. Introduction

The regulation of SP-A expression has been extensively studied. Due to its relatively large molecular mass for a surfactant protein and its hydrophillic nature, it has been relatively easy to both isolate and generate specific antibodies against SP-A. These antibodies have been used to examine the developmental pattern of SP-A in fetal lung in the rat (Schellhase et al., 1988), human (Liley et al., 1989) and rabbit (Snyder & Mendelson, 1988), and in amniotic fluid in the rat (Katyal & Singh, 1983) and human (Snyder et al., 1988). As discussed in chapter 1, it has been consistently observed in every species examined, using either ELISA or immunohistochemical techniques, that SP-A is first detectable around the time when differentiated type II cells are first observed. The relative concentration of SP-A increases with advancing gestational age. Further studies show that the changes in SP-A content of lung during the perinatal period are accompanied by similar alterations in SP-A mRNA levels (Schellhase et al., 1989; Liley et al., 1989; Boggaram et al., 1988; Connelly et al., 1991; Durham et al., 1993). While our investigations were in progress, studies on SP-A gene transcription in rabbit fetal lung were reported (Boggaram et al., 1988b). SP-A gene transcription was detected in nuclei isolated from 21 day gestation fetuses, a few days earlier than the appearance of SP-A mRNA was noted, and there was a rapid increase between 26 and 28 day gestational rabbit fetal lungs. This pattern basically parallels that of SP-A protein and

mRNA abundances during this same period.

The developmental pattern of SP-C expression during fetal lung maturation is not as well characterized as that of SP-A. Studies have been restricted to the mRNA levels only because the extremely hydrophobic nature of the mature SP-C protein makes it very difficult to raise specific antibodies. SP-C mRNA from several species including the human (Liley et al., 1989), rat (Schellhase et al., 1989) and rabbit (Connelly et al., 1991; Wohlford-Lanene et al., 1992a, 1992b) have been examined and in each case it has been suggested that this mRNA species can be detected prior to the morphological appearance of differentiated type II cells and the developmental increases in surfactant phospholipid and SP-A which occur near term.

The effects of glucocorticoids on the expression of SP-A and SP-C genes have been studied using various experimental systems, including lung explants in the human, rat, and rabbit; continuous tumour cell lines; and maternal treatment with glucocorticoids in the rat followed by examination of SP-A and SP-C protein or mRNA levels in fetal lung (for review see chapter 1; Weaver & Whitsett, 1991; Snyder, 1991). Various concentrations of the hormone and durations of treatment have been employed in these investigations. The overall impression obtained was that the effects of glucocorticoids on SP-C gene expression appears less dramatic than that on SP-A (see also Section 1.4.).

As the cDNAs for rabbit SP-A and SP-C were available, we extended our studies to include SP-A and SP-C in addition to SP-B to obtain a more complete picture of the developmental and hormonal regulation of all three surfactant proteins in our system. Some evidence for regulation of SP-C gene transcription in developing lung and upon

glucocorticoic treatment is also presented.

6.2. Results

6.2.1. Probe specificity.

The rabbit SP-A and SP-C cDNA probes used in these studies have been described in previous publications (Connelly et al., 1991, 1992). Northern blot analysis (Fig. 6.1) revealed that in the rabbit the mRNA for SP-A appears as two bands of 2.0-kb and 3.0-kb. The mRNA for SP-C appeared as a single band of 0.9-kb. These sizes are consistent with the lengths of the derived sequences for these two proteins (Mendelson et al., 1986; Connelly & Possmayer, 1992), as well as the number of mRNA species and the sizes reported previously for the rabbit by Boggaram and Mendelson (1988b) and Connelly et al. (1991).

6.2.2. Developmental patterns of SP-A and SP-C mRNA levels and their gene transcriptional activities.

The relative amounts of the mRNAs for SP-A and SP-C were determined by slot blot hybridization and autoradiogram densitometry using the same RNA samples as for SP-B gene expression in chapter 5. Results obtained with lung tissues from fetuses of 22, 24,26,28, and 30-days gestation and neonate 1 day are shown in Fig. 6.2. The results are expressed relative to adult values. SP-A mRNA could not be detected in lungs of 22 days gestation, but was detectable at a very low level on 24 days gestation. The levels then increased markedly between 26-days and 28-days gestation and reached

Figure 6.1. Northern blot analysis of SP-A and SP-C mRNAs in adult rabbit lung tissue. Ten micrograms of total RNA isolated from adult rabbit lung tissue was separated, then hybridized with ^{32}P-labelled rabbit SP-A or SP-C probe. Position of the 18S rRNA is shown (arrow). A single 0.9 kb SP-C mRNA species and two species of SP-A mRNA were detected.

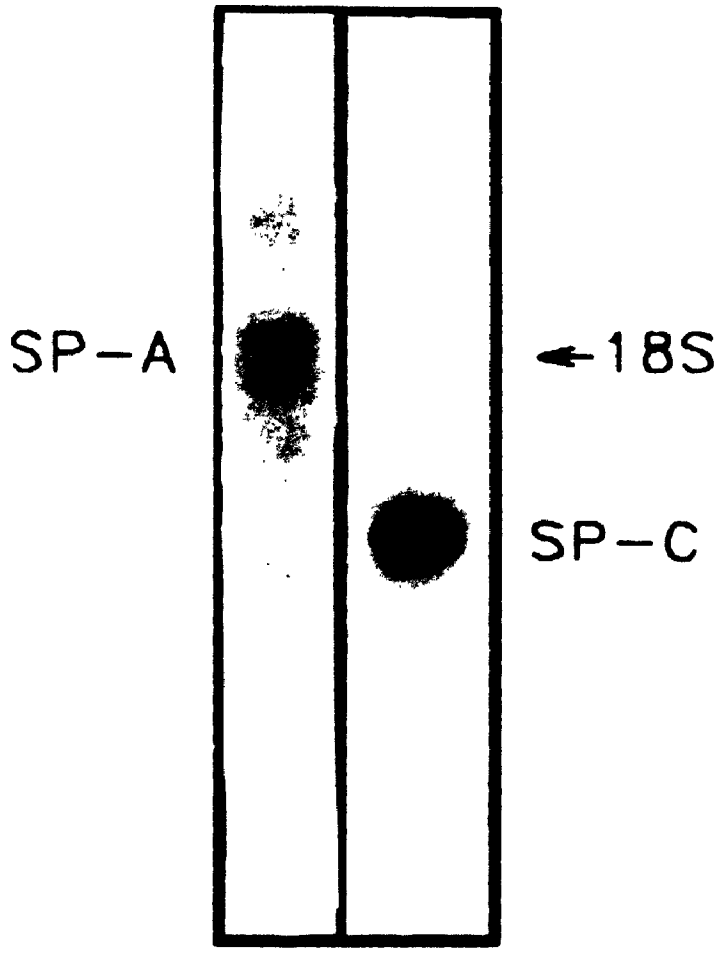
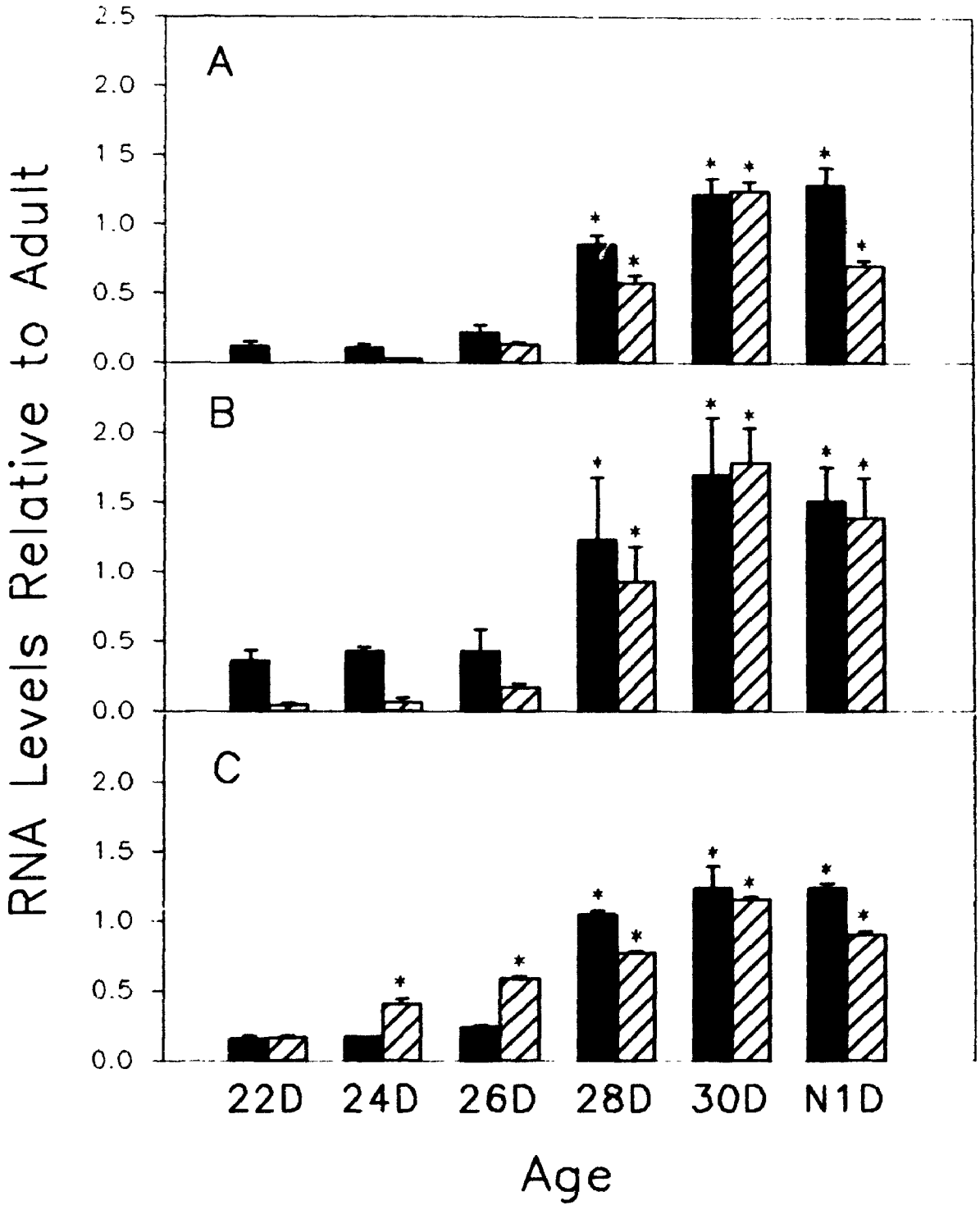

SP-A
18S
SP-C

Figure 6.2. Comparisons of SP-A, SP-B and SP-C gene transcriptional activities and their mRNA levels in rabbit lung during development. The experiments were conducted as in Fig. 4.6. Transcriptional activity *(solid bars)* and mRNA levels *(hatched bars)* are presented as values relative to respective adult levels. Data are the means$\pm$SEM for three or four separate experiments. * indicates values are significantly different from those on 22 or 24 days gestation ($P<0.05$, $n=3$ to 4).


RNA Levels Relative to Adult
A
B
C
2.5
2.0
1.5
1.0
0.5
0.0
22D
24D
26D
28D
30D
N1D
Age

a peak on 30-days (the last day of gestation examined). SP-A mRNA levels were slightly above that of adult at 30-days gestation then fell after birth. SP-C mRNA showed a similar pattern with the levels increasing with advancing gestation. However, this mRNA species differed from SP-A and SP-B mRNA during development in that it was readily detected on day 22 of gestation, the earliest day examined in this study, and increased rather gradually thereafter.

To determine whether the changes in the levels of SP-A and SP-C mRNA during development are regulated at the transcriptional level, the transcription rates of the SP-A and SP-C genes were examined in nuclei isolated from the same lung tissues used for the mRNA slot blot analysis. It can be seen (Fig.6.2.) that transcription of the SP-A and SP-C genes was readily detectable in nuclei isolated from 22 day fetal lung and gradually increased until 26 days of gestation, at which time it reached levels approximately double the levels at 22 days. With both genes there was a marked increase in transcription between 26 days and 28 days gestation. Transcription levels reached a maximum in nuclei from 30 day fetal lung (approx. 6.5-fold increase over day 22 for the SP-A gene and 5-fold for the SP-C gene) and remained at this same level after birth.

Comparison of the transcriptional activities of the genes to their corresponding mRNA levels during development revealed a similar overall pattern for SP-A, SP-B and SP-C, except SP-C mRNA levels were relatively higher at early stages of lung maturation. This latter observation is consistent with that presented by an in situ hybridization study (Wohlford-Lanene et al., 1992a) in which SP-C mRNA was detectable in epithelial cells of the pre-alveolar region of day 19 gestational age fetal

rabbit lung tissue.

6.2.3. Effect of various concentrations of dexamethasone on the levels of SP-A and SP-C mRNA and gene transcription.

In order to examine the dose of dexamethasone that affects the induction and/or accumulation of SP-A and SP-C mRNA in fetal rabbit lungs at early and late stages of lung maturation, lung tissues of day 26 and day 30 gestations were examined in cultures using identical conditions as described in section 5.2.2. The mRNA levels of SP-A and SP-C and their gene transcriptional activities were measured by slot blot analysis, and nuclear run-on assays, respectively, as indicated in chapter 5. The same RNA and nuclei samples were used as in the study of SP-B gene expression.

Exposure of lung explants from fetuses of 26 days gestation to dexamethasone at concentrations from 10^{-10} M to 10^{-6} M led to a 2-5 fold increase in SP-A mRNA levels and a 5-6 fold increase ($p<0.05$) in SP-C mRNA levels relative to tissues incubated in the absence of steroid (Fig. 6.3.), whereas an increase from 3-8 fold was observed for SP-B mRNA (see section 5.2.2). There was, however, no clear dose response to the hormone for SP-A and SP-C mRNAs at 26 days gestation. At the higher concentrations used in this study, dexamethasone produced an approximately 2-fold increase in both SP-A and SP-C gene transcription at this particular gestational age, although the effects were only significant on SP-C gene transcription. This is in contrast with SP-B gene transcription where no apparent effect of dexamethasone on gene transcription was observed (see Section 5.2.2.).

Figure 6.3. Dexamethasone dose response for SP-A, SP-B and SP-C gene transcription and mRNA levels in 26-day fetal lung explants. Experiments were conducted as in Fig. 5.2. Transcription activity *(solid bars)* and mRNA levels *(hatched bars)* are presented as values relative to control (means$\pm$SEM, n=3). * indicates values are significantly different from control values ($P<0.05$).

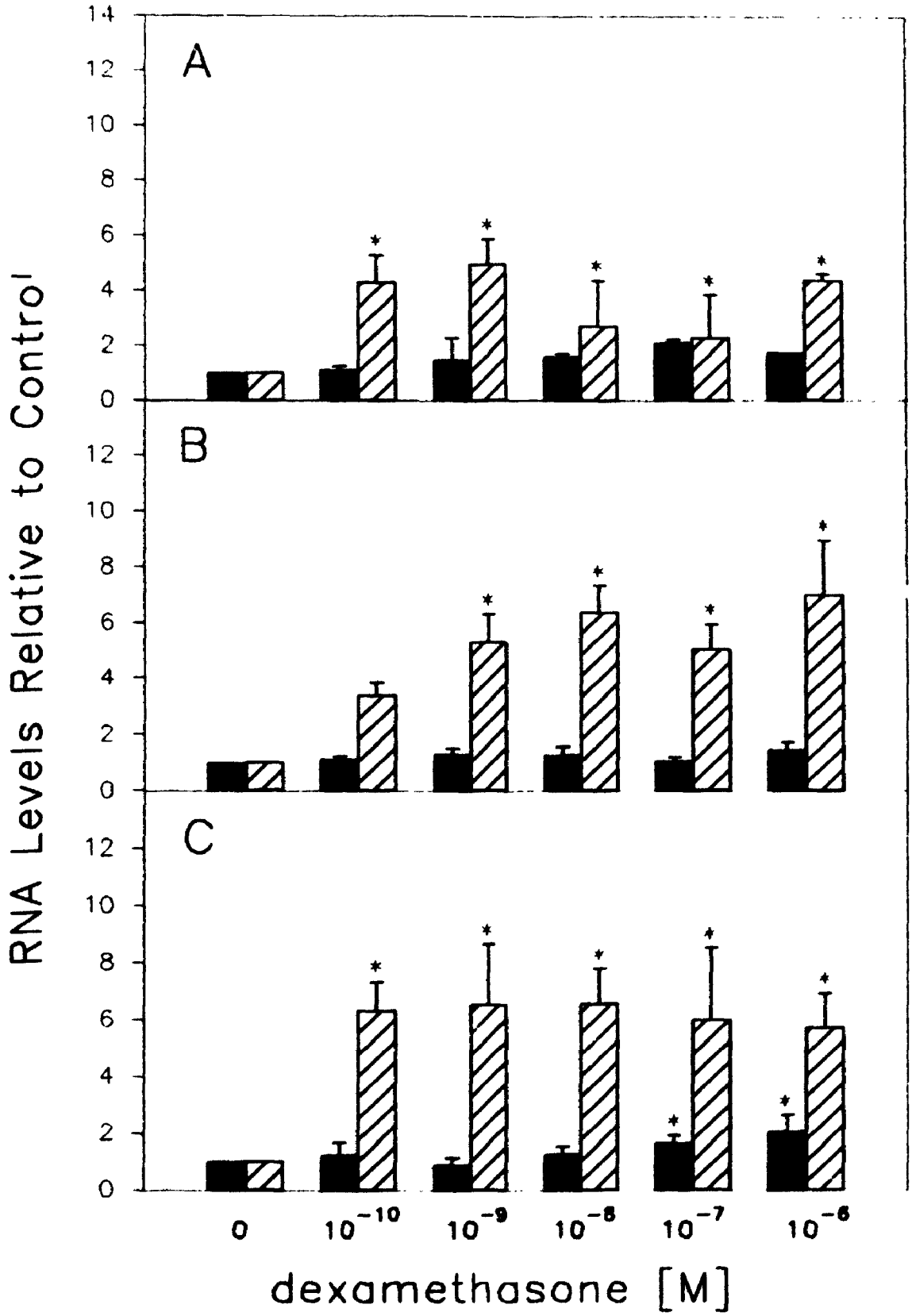
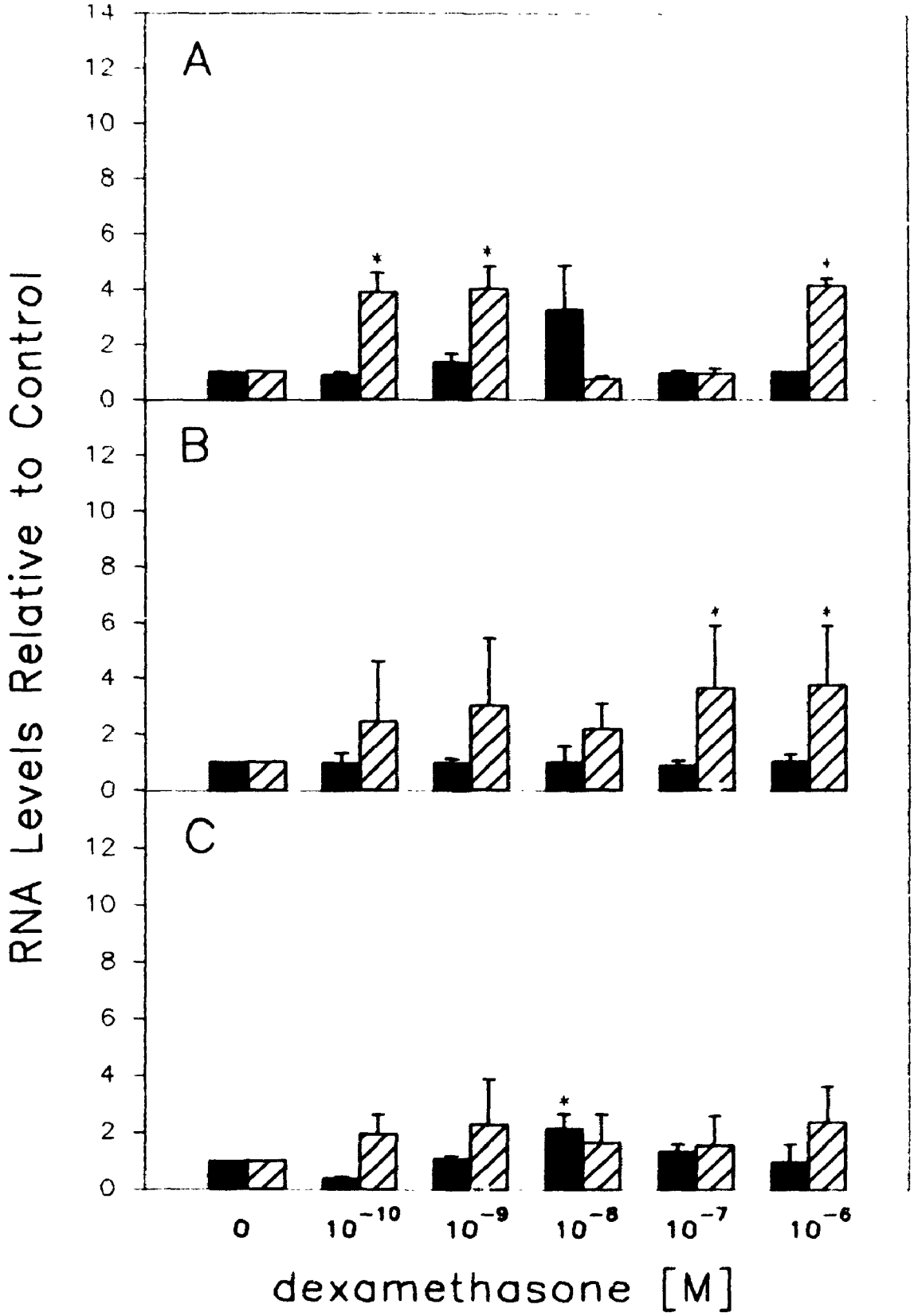

A
B
C
RNA Levels Relative to Control
0
2
4
6
8
10
12
14
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
dexamethasone [M]

Figure 6.4. Dexamethasone dose response for SP-A, SP-B and SP-C gene transcription and mRNA levels in 30-day fetal lung explants. Experiments were conducted as in Fig. 5.2. Solid bars represent transcriptional activity and hatched bars the mRNA levels. Data are means$\pm$SEM with n=3. * indicates values are significantly different from control values ($P<0.05$).


RNA Levels Relative to Control
A
B
C
0
2
4
6
8
10
12
14
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
dexamethasone [M]

With explants from 30 days gestation, increasing the dexamethasone concentration from 10^{-10}-10^{-6} M led to only a modest elevation in SP-C mRNA levels (Fig.6.4). The effects on SP-C gene transcription were small and not significant, similar to those observed with the SP-B gene at this age. In contrast, there were marked changes in SP-A mRNA levels. Increasing dexamethasone concentrations from 10^{-10}-10^{-8} M caused a dose-dependent elevation in SP-A gene transcription. Higher concentrations (10^{-7}-10^{-6} M) led to a fall in SP-A gene transcription to near control levels. SP-A mRNA responded to dexamethasone at concentrations of 10^{-10}M and 10^{-9}M as indicated by a 3-fold increase ($P < 0.05$) over control mRNA levels. At 10^{-8}M the hormone produced a small fall in SP-A mRNA level compared to that of control. In contrast, gene transcriptional activity was elevated but this was not significant. SP-A mRNA and gene transcription were both near control levels in explants cultured with 10^{-7}M dexamethasone. At concentrations of 10^{-6}M, the steroid had a stimulatory effect ($P < 0.05$) on SP-A mRNA levels whereas gene transcription remained at the control value.

6.2.4. Effects of dexamethasone on SP-A and SP-C gene transcription and their mRNA levels as a function of incubation time.

The effects of dexamethasone on the regulation of the abundance of SP-A and SP-C mRNAs were further investigated by culturing lung explants for up to 7 days with hormone at 10^{-8} M added after the first 24 h in culture as in Section 5.2.3. With explants from fetuses of 26 days gestation cultured in the absence of dexamethasone (Fig.

Figure 6.5. Day-26 lung SP-A, SP-B and SP-C mRNA and run-on transcript levels in response to dexamethasone treatment. The experiments were conducted as in Fig.5.3. Dashed lines and open symbols indicate data obtained from explants incubated in control medium whereas solid lines and closed symbols were from cultures containing dexamethasone at 10^{-8}M. The transcriptional activities of the gene are shown by the triangles and the mRNA levels by the circles. Data are means$\pm$SEM with n=3. * indicates values are significantly different from respective control values ($P<0.05$).

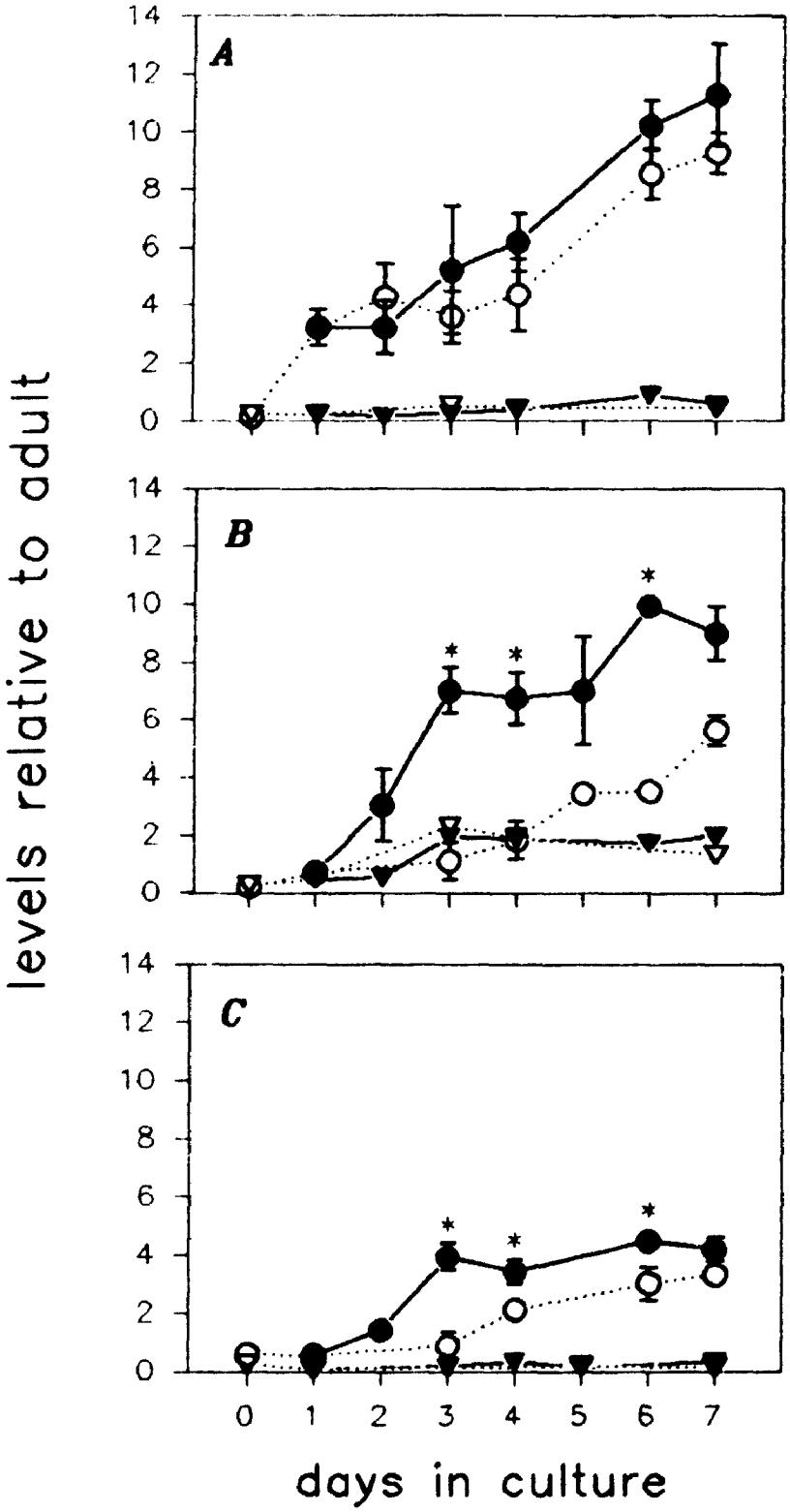
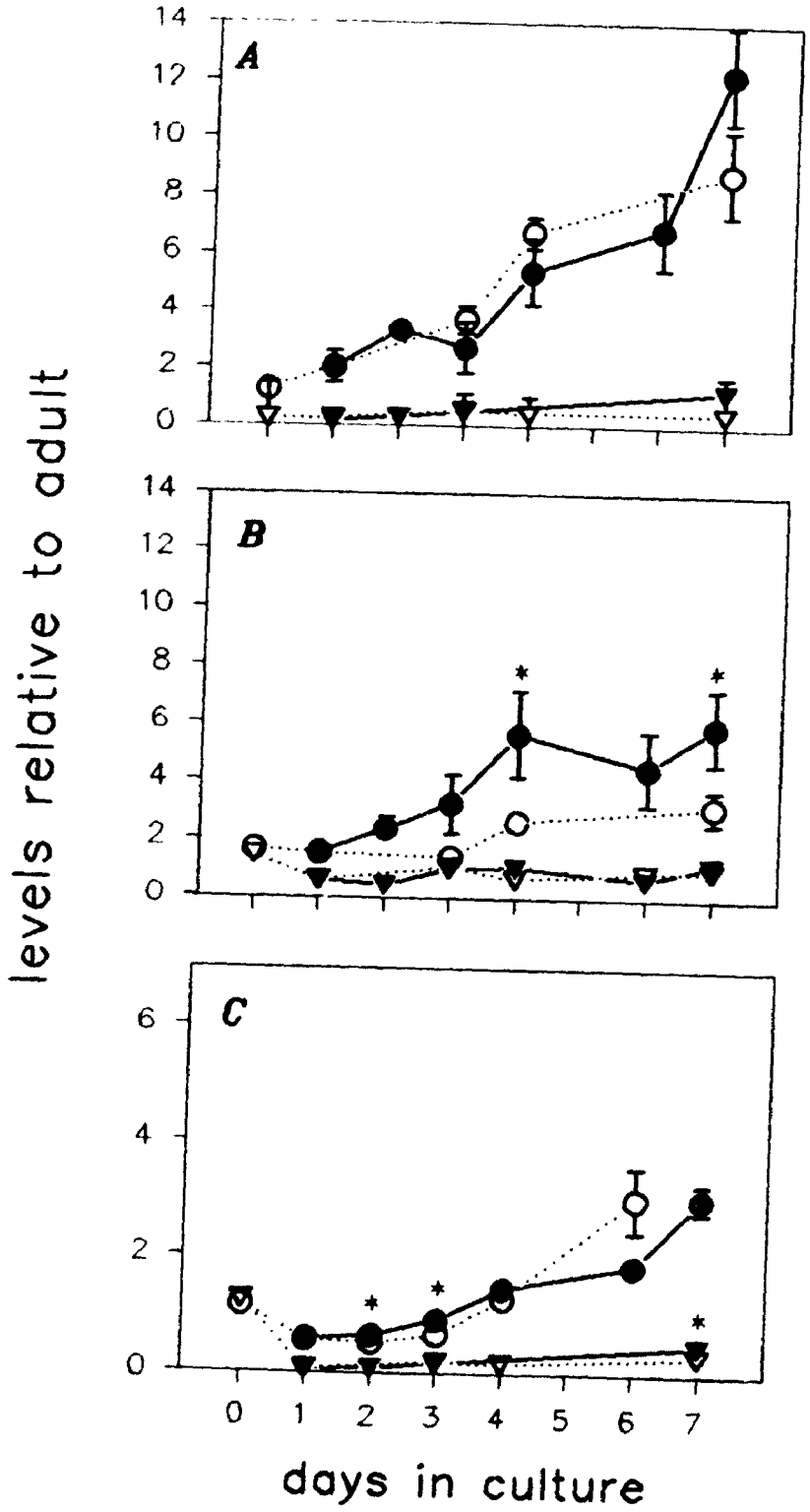

A
B
C
14
12
10
8
6
4
2
0
*
0 1 2 3 4 5 6 7
days in culture
levels relative to adult

Figure 6.6. Day-30 lung SP-A, SP-B and SP-C mRNA and run-on transcript levels in response to dexamethasone treatment. The experiments were conducted as for Fig. 5.4. and 6.5. Triangles represent the gene transcription and circles represent the message levels. Closed symboles indicate data obtained with lung explants in the presence of dexamethasone and open symboles in the absence of the hormone. Data are means$\pm$SEM with n=3. * indicates values are significantly different from respective control values ($P<0.05$).


A
B
C
levels relative to adult
days in culture
0
2
4
6
8
10
12
14
0 1 2 3 4 5 6 7
*

6.5), SP-A mRNA levels exhibited a rapid increase during the first day in culture, followed by a gradual elevation which accelerated mainly between days 4 and 7, resulting in levels approximately 8-fold the adult level. SP-C mRNA content was not altered in culture until day 3 when an increase became apparent and the levels accelerated gradually thereafter yielding a final level approximately 3-fold the adult value. The presence of dexamethasone (10^{-8}M) produced a rapid increase ($P < 0.05$) in SP-C mRNA steady-state levels to ~4 fold adult level within 48 h of treatment and it remained at the same level thereafter, a similar overall pattern to that observed with SP-B mRNA abundance. In contrast, dexamethasone at this concentration caused merely a small increase in SP-A mRNA levels over control.

The naturally occurring changes in the SP-A mRNA levels (i.e., no dexamethasone) observed in fetal lung cultures of 30 days gestation (Fig. 6.6) showed fairly similar patterns compared to that observed with tissues of 26 days gestation. This was true for both the magnitude and the duration of the changes. At this gestational age, the hormone again had no discernible effect. SP-C mRNA levels in fetal lung of 30 days, in contrast, showed a somewhat different profile from that observed with 26 days lung tissue. Incubation in the absence of dexamethasone led to a slow moderate increase in SP-C mRNA throughout culture. Addition of steroid at 10^{8} M resulted in a very small increase in the mRNA levels. The SP-C mRNA levels increased gradually to levels ~3-fold of adult on day 6 or day 7 of culture in the presence and absence of dexamethasone. Thus, dexamethasone at 10^{-8}M showed similar stimulatory effects on SP-B and SP-C mRNA levels in 26-day lung explants and these effects were less evident

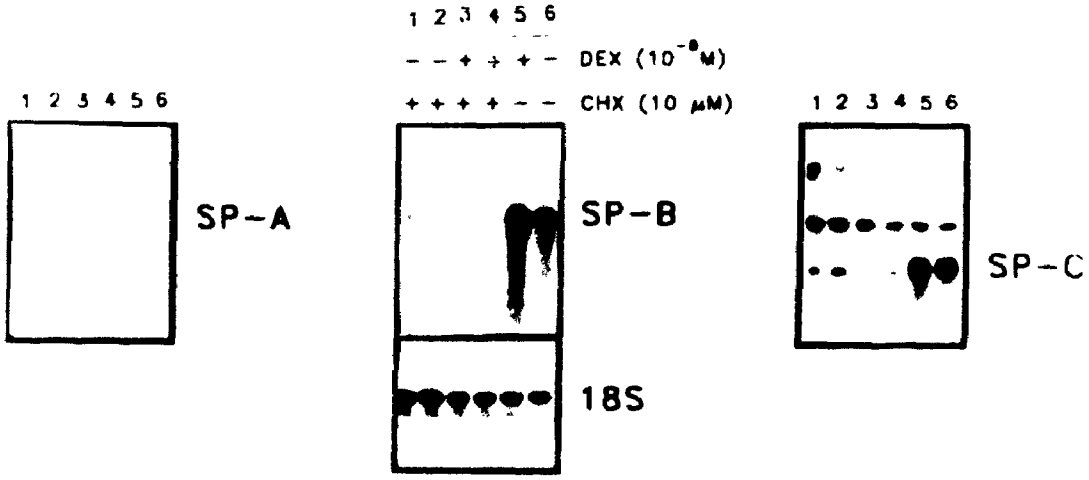
with SP-C mRNA levels in 30-day lung explants. At the concentration employed, the hormone did not seem to have any significant effects on SP-A mRNA content with lung explants of either gestational age.

To examine whether dexamethasone had any effect as a function of culture time on the transcription activities of the SP-A and SP-C genes, relative transcription rates of the two genes were measured by nuclear run-on analysis. Initially, transcription rates of the SP-A and SP-C genes were low in fetal lung of 26 days gestation (Fig. 6.5; see also Fig. 6.2.) and remained at similar levels during culture. Addition of dexamethasone at 10^{-8} M did not produce any further increase in the transcription rates of the genes for either surfactant protein. With explants from 30 days gestation lungs (Fig. 6.6), the transcription rates of the SP-C gene fell during the first day in culture from levels similar to that of adult lung (see also Fig.6.2.) to low values. Transcription rates of the SP-A gene increased gradually during culture. Transcriptional activities of the SP-C gene also increased during the incubation but the final level was lower than that of preculture. Similar to that found with 26 days lung, dexamethasone at 10^{-8} M did not cause a significant increase in the transcription rates of SP-A and SP-C genes in 30 day gestation lung explants except possibly with SP-C on day 7 in culture. These results were similar to those observed with the SP-B gene transcription.

6.2.5. Effects of cycloheximide on SP-A and SP-C mRNA production and accumulation in the presence or absence of dexamethasone.

In addition to examining the effects of cycloheximide on the dexamethasone-

Figure 6.7. Effects of cycloheximide and dexamethasone alone or in combination on SP-A, SP-B and SP-C gene transcription and mRNA levels in 26-day fetal lung explants. The experimental protocol shown for SP-B (Fig. 5.7) applies to all the other panels in this figure. Representative autoradiograms of Northern analysis of the mRNA are shown in the upper panels and nuclear run-on analysis in the lower panel. Note that in the Northern blot probed with SP-C, a band is seen above the SP-C signal. This was due to incomplete stripping of the previously applied 18s cDNA probe.


1 2 3 4 5 6
SP-A
1 2 3 4 5 6
– – + + + – DEX (10^{-6}M)
+ + + + – – CHX (10 μM)
SP-B
18S
1 2 3 4 5 6
SP-C

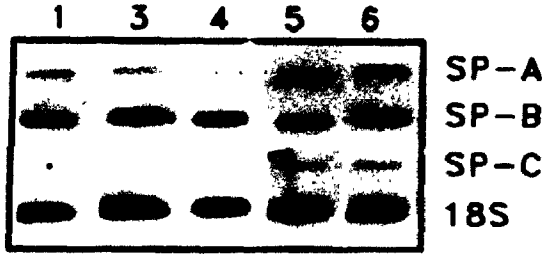
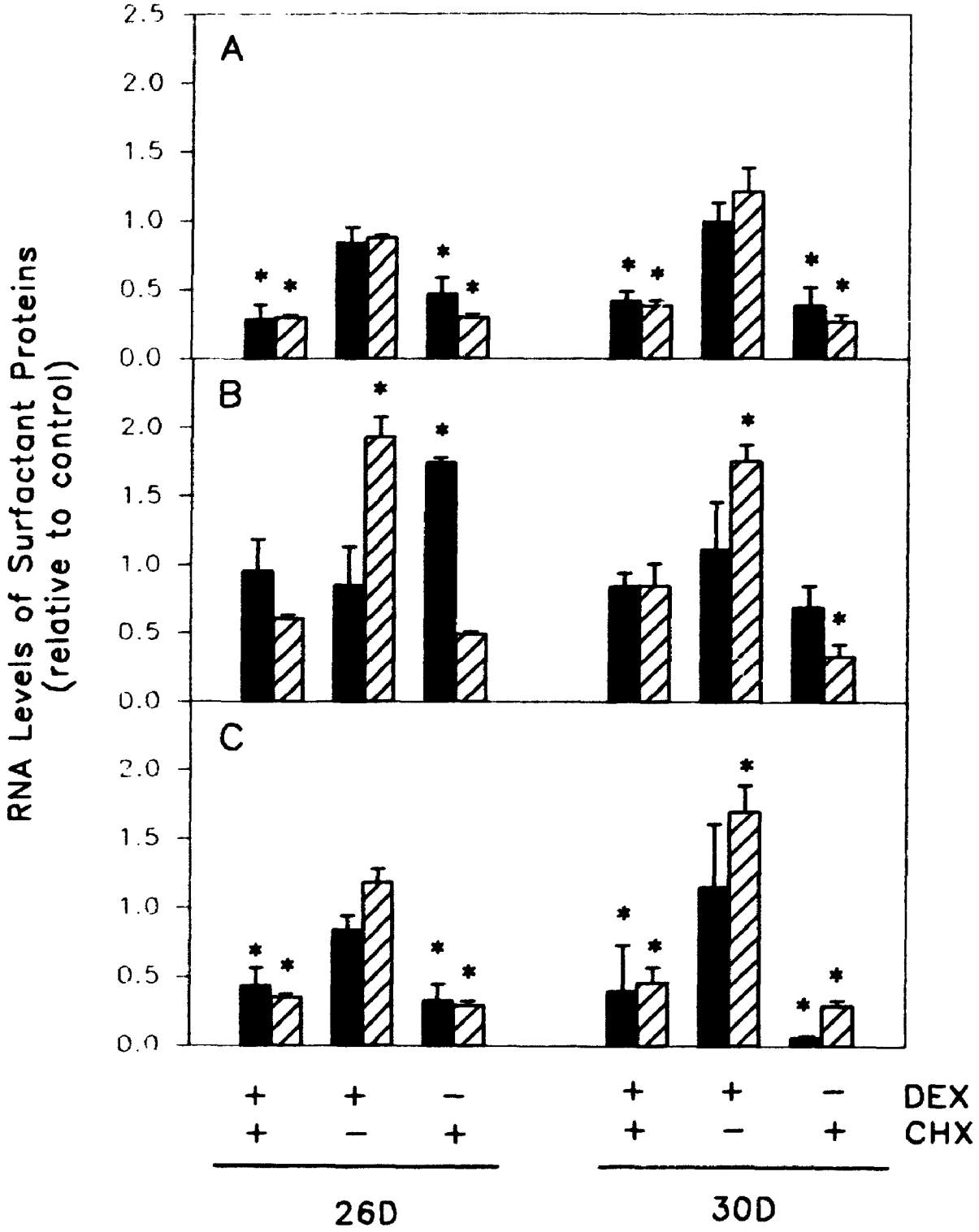

1 3 4 5 6
SP-A
SP-B
SP-C
18S

Figure 6.8. Summary of the effects of cycloheximide and dexamethasone alone or in combination on gene transcription and mRNA levels of SP-A, SP-B and SP-C (Fig. 6.7). Data (means$\pm$S.E., n=3) were obtained by densitometry of the autoradiograms shown in Fig. 6.7 except the mRNA levels were measured by slot blot analysis. Solid bars represent transcriptional activities of the genes and hatched bars the message levels.


RNA Levels of Surfactant Proteins
(relative to control)
2.5
2.0
1.5
1.0
0.5
0.0
A
B
C
+ + − + + −
+ − + + − +
DEX
CHX
26D
30D

induced increase in SP-B mRNA levels, the effects of this inhibitor on SP-A and SP-C mRNA accumulation were also studied in explants in the presence or absence of dexamethasone. Lung explants from 26-day and 30-day fetal rabbits were treated for 24 h with 10^{-8}M dexamethasone (added on day 1 of culture) in the presence or absence of 10 μM cycloheximide and the levels of the mRNAs for SP-A and SP-C and their gene transcriptional activities were determined. As shown in Fig. 6.7 and 6.8., dexamethasone (10^{-8}M) had no detectable effect on the synthesis or accumulation of SP-A mRNA in 26-day explants (see also Fig. 6.5); however, slightly increased SP-A mRNA levels (1.2-fold compared to control) but not SP-A gene transcription in 30-day explants were noted. In both 26-day and 30-day explant tissues incubated with cycloheximide (10 μM) alone, the levels of SP-A gene transcription were reduced to less than half of that detected in untreated explants. This was accompanied by a similar decrease in SP-A mRNA level. Essentially identical patterns were observed when the explants were cultured in medium containing both dexamethasone and cycloheximide.

In the case of SP-C mRNA levels, dexamethasone (Fig. 6.7 and 6.8) at 10^{-8}M increased the mRNA levels 1.2-fold and 1.7-fold in 26-day lung and 30-day lung explants, respectively. There was no significant effect of the hormone on the SP-C gene transcription in explants of either gestational ages, as shown in Fig. 6.5. Similar to that observed with SP-A mRNA, cycloheximide at 10 μM alone caused decreases in both the synthesis and accumulation of SP-C mRNA to about 50% of that determined with untreated explants. The effect was more pronounced in 30-day explants than in 26-day explants. Combined cycloheximide and dexamethasone treatment resulted in identical

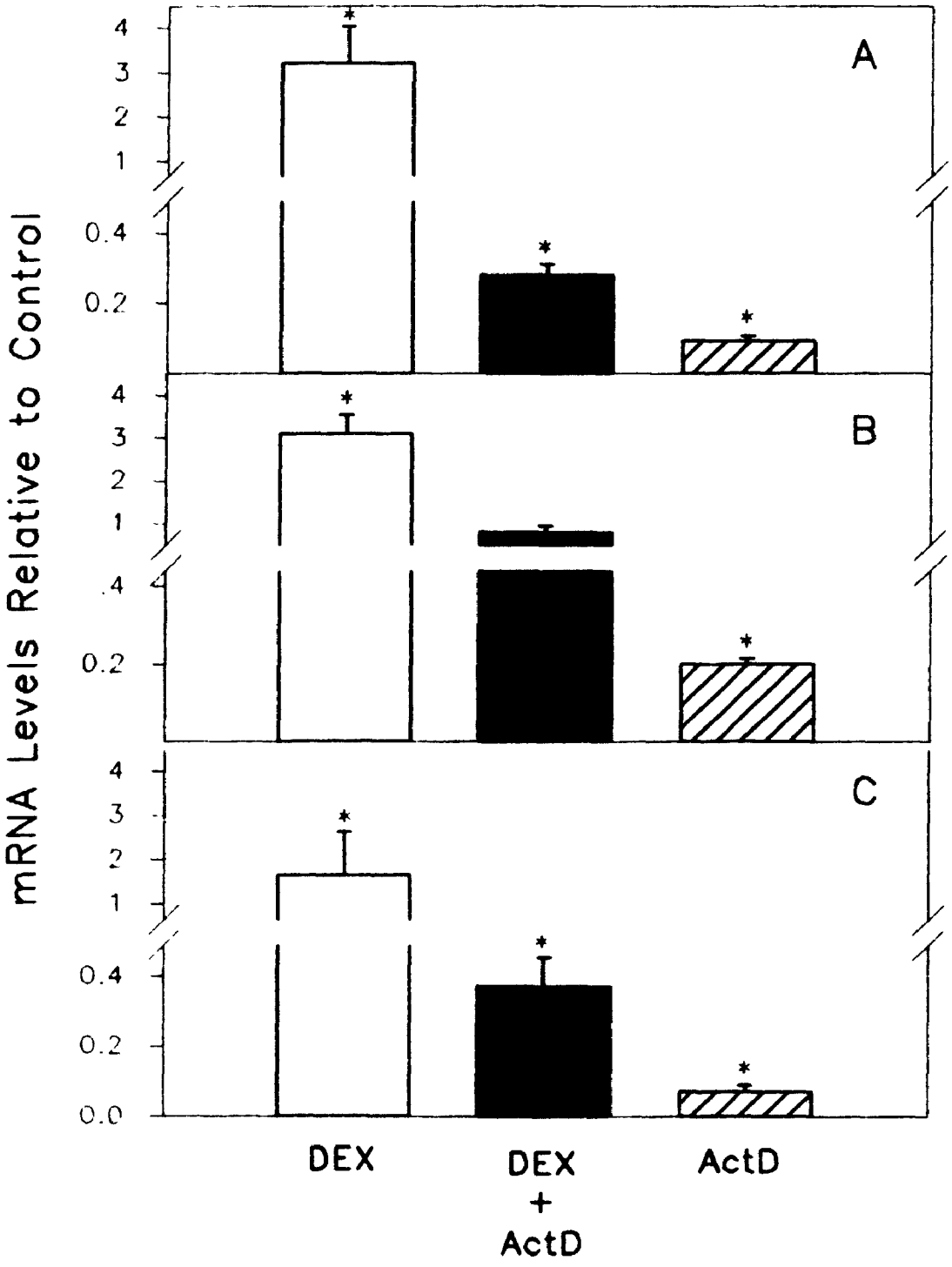
alterations in SP-C mRNA levels and gene transcription as those observed with the inhibitor alone. It should be noted that cycloheximide treatment did not have a significant inhibitory effect on the overall transcriptional activity of fetal lung nuclei nor on the rate of transcription of 18s RNA or 18s RNA levels in lung explants (Fig. 6.7).

The effects of cycloheximide alone, or in combination with dexamethasone, on SP-A and SP-C mRNA levels were considerably different from those on SP-B mRNA. A decrease in SP-B mRNA levels but no change or even an elevation in the SP-B gene transcription was observed (see Section 5.3). Taken together, these finding suggest that labile protein factors are required for (1) the regulation of the transcriptional activities of the SP-A and SP-C genes; and (2) the post-transcriptional regulation of all three surfactant-proteins mRNA levels.

6.2.6. Effects of actinomycin D on SP-A, SP-B and SP-C mRNA levels in the presence or absence of 10^{-8}M dexamethasone.

It has been reported that SP-A mRNA levels in human fetal lung explants were reduced by incubation with dexamethasone at 10^{-7}M (Boggaram et al., 1989) and this was attributed to a reduction in the apparent half-life of the mRNA by the hormone (Boggaram et al., 1991). Although no pronounced effect of dexamethasone (10^{-8}M) on SP-A mRNA levels was observed in the present study, the effects of actinomycin D (5 μM) alone or in combination with dexamethasone on SP-A and SP-C mRNA abundance were investigated In the study shown in Fig. 6.9., rabbit fetal lung explants were treated for 24 h with 10^{-8}M dexamethasone (added on day 1 of culture) in the presence

Figure 6.9. Effects of dexamethasone (DEX) on SP-A, SP-B and SP-C mRNA levels in the presence and absence of actinomycin D (ActD). Experiments were conducted as for Fig. 5.7. Data are presented as means$\pm$SEM with n=3. * indicates values are significantly different from control values ($P<0.05$).


mRNA Levels Relative to Control
A
B
C
4
3
2
1
0.4
0.2
0.0
*
DEX
DEX + ActD
ActD

or absence of 5 μM actinomycin D. Total RNA was isolated from the explant tissues and SP-A and SP-C mRNA levels were determined as for SP-B mRNA (chapter 5). In this study, incubation with dexamethasone of 10^{-8}M for 24 h resulted in a 3-fold increase vs control in SP-A and SP-B mRNA levels and a 2-fold increase in SP-C mRNA levels. Addition of 5 μM actinomycin D to control medium for 24 h caused marked reductions in both SP-A and SP-C mRNA levels. SP-A mRNA was reduced by 90% and SP-C mRNA was barely detectable. When dexamethasone (10^{-8}M) was added at the same time as actinomycin D, SP-A and SP-C mRNA were retained at higher levels ($P<0.05$) although the level was still less than half of that in control explants, whereas SP-B mRNA remained at levels near control values. These findings suggest that the effect of dexamethasone at this concentration on post-transcriptional regulation of SP-A and SP-C mRNAs is not as apparent as on that of SP-B mRNA.

6.3. Discussion

The first part of this chapter describes studies on the ontogeny of SP-A and SP-C mRNA levels and their relative gene transcription rates in developing rabbit lung. While the pattern of SP-A gene transcription in rabbit fetal lung has been presented previously by Boggaram et al. (1988), this is the first report on the developmental pattern for SP-C gene transcription. We observed that gene transcription for rabbit SP-A and SP-C was readily detectabie at low levels in 22- and 24-day rabbit fetal lung, increased markedly between 26 days and 30 days gestation and remained at the same level after birth.

The developmental pattern for SP-A gene transcription rates was essentially

identical to that observed in fetal rabbit lungs by Boggaram and Mendelson (1988b). In their study, it was observed that SP-A gene transcription was first detectable on day 21 of gestation. Transcription rates increased with advancing gestation, reaching a maximum level in lung nuclei from 28-day fetal rabbits. We observed in the present study that SP-A gene transcription was detectable in lung nuclei from 22 day fetal rabbits, the earliest age examined, and reached a maximum at 30 days of gestation. The difference may be due to the incomplete sampling of the other researchers who did not examine samples between 28 days gestation and term. We also observed that SP-A gene transcriptional activity remained similar in nuclei isolated from 30 day gestational age and neonatal 1 day old rabbits. This is in disagreement with the results cited above (Boggaram & Mendelson, 1988) which showed that the levels of SP-A gene transcription declined slightly in neonates, although the age of neonates used in the study was not specified.

The ontogeny of SP-A mRNA levels has been examined in fetal rabbit lung by this laboratory (Connelly et al., 1991) and by some other investigators (Boggaram et al., 1988; Durham et al., 1993). In the present study, SP-A mRNA was first detected at a very low level by slot blot analysis in rabbit fetal lung of day 24 of gestation, the same gestational age as it could be detected by Northern blot analysis (Durham et al., 1993). Boggaram et al. (1988), also using Northern blot analysis, first detected SP-A mRNA in fetal rabbit lung tissue on day 26 of gestation. The discrepancy in the onset of SP-A mRNA in fetal rabbit lung is probably due to technical reasons, e.g., the sensitivity of different assays or the manner in which the techniques were applied. Connelly et al.

(1991) in this laboratory reported that by using a more sensitive solution hybridization assay, SP-A mRNA could be detected in fetal rabbit lungs as early as day 22 of gestation. Augmented accumulation of SP-A mRNA in rabbit fetal lung occurred only after 26-days of gestation. This is in agreement with the observation that SP-A mRNA and protein can be localized at 25 or 26 days gestation in epithelial cells of the pre-alveolar region of rabbit fetal lung tissues by in situ hybridization (Wohlford-Lanene et al., 1992b; Auten et al., 1990) or immunochemistry (Snyder and Mendelson, 1987). The different gestational ages reported with respect to the time SP-A mRNA was first detectable fall into the same stage of lung development, i.e., the last third of the pseudoglandular phase (Fig. 1.5). Thereafter, SP-A mRNA increased dramatically throughout the remainder of gestation and declined in the neonate to lower levels than observed at 30 or 31 day gestation (Connelly et al., 1991; Boggaram & Mendelson, 1988a; Wohlford-Lanene et al., 1992b). The ontogenic profile at the later stages of lung development for SP-A mRNA observed in this study was consistent with studies published previously.

SP-A mRNA levels during perinatal development have also been examined in the rat (Schellhase et al., 1988), and human (Liley et al., 1987). SP-A mRNA was first detected in rat fetal lung at day 18 of gestation (term 22 days), within the last third of the pseudoglandular phase of lung development. In contrast, SP-A mRNA was not detected in human lung as late as 24 weeks of gestation, a time point well into the canalicular or possibly even within the saccular phase of lung development. The reason for the difference between the onset time for SP-A mRNA expression in fetal lung of the

rodent and the human is not clear.

Interestingly, SP-C mRNA ontogeny in the rabbit showed a different temporal pattern from that of SP-A or SP-B (chapter 5). SP-C mRNA levels were detected at relatively high levels as early as 22 days of gestation. By 26 days, it increased to more than half of the adult level and then accelerated gradually until it peaked at 30 days of gestation and fell slightly in the neonate. The dramatic increase in the levels of SP-A and SP-B mRNA observed between days 26 and 28 of gestation was not as apparent with SP-C mRNA. An almost identical pattern for SP-C mRNA levels in this species was also observed by Connelly et al. (1991) and Wohlford-Lanene et al. (1992) . In contrast, SP-C gene transcriptional activity, which has not been described previously in the rabbit, showed a similar pattern to that for SP-A and SP-B gene transcription. SP-C gene transcription detected in nuclei from 22 day gestation rabbit lung, remained at low levels until 26 days of gestation, increased markedly between 26 and 28 days, peaked at 30 days and then remained at approximately the same level after birth.

SP-C mRNA levels have also been examined in rat (Schellhase et al., 1988) and human lung (Liley et al., 1989). In the rat, a similar temporal pattern was observed as compared to rabbit SP-C mRNA induction during development. SP-C mRNA was first detected in rat at 17 days of gestation, which is in the last third of the pseudoglandular phase of lung development, as in the rabbit. In human fetal lung, SP-C mRNA was detectable at 13 weeks of gestation and increased in relative concentration during the next 10 weeks (Liley et al., 1989). The appearance of human SP-C mRNA, although temporally as early as 33% of the length of gestation, occurs at the same stage of lung

morphological development as in the rat and the rabbit, the last third of the pseudoglandular phase.

Our data with respect to the induction of the mRNAs for the surfactant-associated proteins, SP-A and SP-B, during rabbit fetal lung development are consistent with the induction of SP-A and SP-B mRNA preceding the differentiation of alveolar type II cells by a few days, as suggested by the studies cited above. In contrast, significant amounts of SP-C mRNA are present in rabbit fetal lung tissue several days prior to the appearance of differentiated type II cells. Nevertheless, both the pattern and magnitude of the induction of all three surfactant protein mRNAs, whether described as accumulation or synthesis of mRNA, showed strong similarity during the later stages of lung development (after 26 days of gestation) (see Fig. 6.2), supporting the conclusion that the three genes are regulated differently but are somehow coordinately induced as the fetus approaches term (Connelly et al., 1991). This coordination at the mRNA level could occur in the rabbit specifically in type II cells, since this coordinating event did not happen until type II cells begin to differentiate on day 26 of gestation (Snyder et al., 1991; Also see Section 1.2.2.2).

Before 26 days of gestation in the rabbit, different patterns of gene expression for the three surfactant proteins were observed in this study. SP-A and SP-B mRNA levels were very low on days 22 and 24 of gestation, whereas SP-C mRNA was already present at a relatively high level compared to the adult. Our data are suggestive that in the rabbit the developmental regulation of the mRNA levels of SP-A and SP-B is different from that of SP-C. It has been shown by in situ hybridization studies that SP-C mRNA was

present in all epithelial cells of the pre-alveolar region of day 19 gestational age rabbit fetal lung tissue (Wohlford-Lanene et al., 1992a). This could contribute to the relatively high levels of SP-C mRNA detected in whole lung of day 22, 24 and 26 days gestation rabbits (Fig.6.2 and Wolhford-Lanene et al., 1992a). Whether the presence of this mRNA in the lung is of any physiological significance is not known. Due to the lack of specific antibodies directed against SP-C, no direct evidence has been obtained indicating that SP-C mRNA detected during the early stage of lung development was translated into SP-C protein.

On the other hand, SP-B gene transcription was consistently detected at a relatively high level during this early stage in lung development. The basis of this high transcriptional activity of SP-B gene is not clear. In situ hybridization in developing fetal rabbit lung (Wohlford-Lanene et al., 1992b) has demonstrated that SP-B mRNA can be localized in alveolar type II cell precursors on day 26 of gestation (the earliest day examined). SP-B mRNA concentration increased throughout the remainder of gestation. SP-B mRNA was also detected in bronchiolar epithelial cells but not until day 28 of gestation. The concentration in this cell type also increased during gestation. Thus, SP-B mRNA appears restricted to only type II cell precursors at the early stages of lung development. The significance of the relatively high transcriptional activity of SP-B gene during this period is not fully understood.

The similar developmental profiles observed for SP-A protein and mRNA in fetal human (Ballard et al., 1986; Whitsett et al., 1987; Liley et al., 1989) and rat (Schellhase et al., 1991; Shimizu et al., 1991) lung or in amniotic fluid (Snyder et al., 1986)

suggested that the control of expression of this protein in vivo is primarily pretranslational. This manner of regulation is also suggested for SP-B protein in that the ontogenic pattern for SP-B protein and its mRNA in the rat could also be aligned to one another (Shimizu et al., 1991; Schellhase et al., 1988). The fact that SP-A, SP-B and SP-C mRNA levels parallel that of their respective transcriptional activities implies that starting from 28-days of gestation in the rabbit, the in vivo mRNA levels of SP-A, SP-B and SP-C could be controlled mainly at the transcriptional level. The different patterns observed in the rabbit in vivo before 26-days gestation with SP-A, SP-B and SP-C mRNA levels and their gene transcriptional activities in the rabbit suggest different mechanisms may be involved in the regulation of surfactant protein mRNA levels during the early stages of lung development. More sophisticated approaches are needed to examine these hypotheses. Cis-acting regions conferring lung epithelial cell-specificity and regulation of developmental expression have been identified in the human SP-C gene and partially characterized in transgenic mice (Glasser et at., 1991). In this study, the transcriptional activity of the SP-C gene, detected by examining the chimeric SP-C-CAT (chloramphenicol acetyltransferase) activity increased dramatically between 13-14 days of gestation (term 22 days), indicating the potential of transcriptional regulation of the SP-C expression during the early stage of lung development in the mouse (Glasser et al., 1991).

As stated in section 1.4., cis-acting elements cannot exert their actions without the existence of trans-acting factors which are triggered or produced at certain stages in the cell cycle or under the presence of certain stimuli. Glucocorticoids, as a stimulus,

can be a cause for many of these events to occur in a living cell, tissue or organ by binding to the specific receptors followed by translocation of the hormone-receptor complex into the nucleus (see section 1.4.1). Glucocorticoid regulation of surfactant protein SP-A expression during the perinatal period has been extensively studied (for review see Snyder, 1991; Weaver and Whitsett, 1991; Post, 1992). In our study, we extended our examination of SP-B gene expression (chapter 5) to that of SP-A in fetal rabbit lung. In contrast to SP-B expression where dexamethasone caused a significant increase in mRNA levels but not gene transcription, we observed in the dose-response study that the hormone appeared to have biphasic effects on both SP-A mRNA levels and its gene transcriptional activity. Increasing the concentrations of dexamethasone from 10^{-10}M to 10^{-7}M resulted in an increase in SP-A gene transcription but a decrease in SP-A mRNA levels. The change in SP-A mRNA levels is consistent with observations obtained in human fetal lung explants in vitro which demonstrated a dose-dependent biphasic effect of dexamethasone (Boggaram et al., 1989). The biphasic effect of dexamethasone occurred with rabbit lung explants of 30-day fetuses (30/31 gestation), whereas those observed with human lung occurred with explants of 15-18 weeks (term 40 weeks) fetuses. This difference could be due to species specificity of the glucocorticoid response. Glucocorticoid treatment of lung explants from 18-day rats produced only a stimulation in SP-A mRNA levels at all doses tested (Nicholes et al., 1990). The biphasic effect of glucocorticoids on 21-day rabbit fetal lung was time-dependent (Boggaram & Mendelson, 1988b). It was shown that the hormone had a transient inhibitory effect on SP-A gene transcription within 24 h of its addition, whereas

a stimulatory effect was observed thereafter. This reduction in SP-A gene transcription was accompanied by a corresponding decrease in SP-A mRNA levels.

In our time-course studies, no significant effect of dexamethasone was observed on either SP-A mRNA steady-state levels or SP-A gene transcriptional activity in rabbit lung explants of either 26- or 30-days gestation fetuses, although the levels of these two parameters increased quite rapidly during the course of culture. The lack of a pronounced response of SP-A mRNA to 10^{-8}M dexamethasone could be due to an inappropriate sampling window with respect to gestational age of the fetus or to the steroid concentration. Dexamethasone at 10^{-8}M has been found to cause 8- to 10-fold increases in SP-A mRNA levels with fetal human lung explants of 15-18 weeks gestational age (Liley et al., 1989). In addition, previous studies which provided evidence for the effects of glucocorticoids on rabbit SP-A mRNA synthesis and accumulation were performed using 21-day fetal lung explants (Boggaram et al., 1988b). The observation that SP-A gene transcriptional activity, as measured in this study, was not affected in lung explants of either 26-day or 30-day fetuses by the addition of dexamethasone indicated that fetal lung tissue might not be as responsive to in vitro glucocorticoid administration at the late stages of perinatal period as at the early stages. This is supported by in vivo studies (Schellhase et al., 1991; Shannon et al., 1991) which showed that maternal administration of dexamethasone at early (17/21 day gestation) gestational ages resulted in an increase in SP-A mRNA and protein contents whereas no change was observed in day-19 fetal lung (19/21 day).

It was also observed in the present study that the acceleration or maintenance of

SP-A mRNA levels during explant culture with both 26-day and 30-day fetal rabbit lungs was dependent on *de novo* RNA and protein synthesis. This is indicated by the fact that actinomycin D reduced SP-A mRNA to very low levels compared to controls. Cycloheximide caused a remarkable reduction in both SP-A mRNA levels and its gene transcription in explants of both ages. These results taken together indicate that SP-A mRNA levels in lung explants of 26-days and 30-days gestations may be regulated mainly at the transcriptional level and that fetal lung explants of these two ages are not very responsive to in vitro dexamethasone (10^{-8}M). The rapid increase in SP-A mRNA levels during explant culture observed in present study implied an involvement of other agents in regulation of SP-A gene expression. In fact, cyclic-AMP and its analogues have been found to be very potent stimulators of SP-A mRNA production (for review see Mendelson and Boggaram, 1991; Snyder, 1991 and Weaver and Whitsett, 1991). Studies of in vivo glucocorticoid treatment in the rat (Shimizu et al., 1991; Schellhase et al., 1991; Shannon et al., 1991; Fisher et al., 1991) in which a significant increase in SP-A mRNA and protein contents were detected also suggest that regulation of SP-A mRNA levels by glucocorticoids may require the presence of other hormones or agents.

During our studies of the regulation of SP-C mRNA levels, we observed in dose-response studies that dexamethasone treatment in vitro resulted in a rapid increase in SP-C mRNA content in 26-day lung explants but a more moderate acceleration in 30-day lung explants. The response to dexamethasone was not dose-dependent at either age. Although this observation was inconsistent with those from human (Liley et al., 1989) and rat (Veletza et al., 1992) fetal lung explants, in our studies SP-C gene transcription

did appear to have a dose-dependent response to dexamethasone (10^{-9}M - 10^{-6}M for 26-day explants and 10^{-10}-10^{-8}M for 30-day explants). These results showed that the regulation of SP-C mRNA levels by dexamethasone could involve a transcriptional control step, a possibility also suggested by an in vitro study in the rat which demonstrated a 2.5-fold increase in SP-C gene transcription in 18-day rat lung explants incubated with 10^{-7}M dexamethasone for 24 h (Veletza et al., 1992). Furthermore, as observed in our study, cycloheximide caused a significant decrease in SP-C mRNA as well as in SP-C gene transcriptional activity. An increase in chimeric SP-C-CAT activity upon dexamethasone treatment of lung explants from transgenic mice (Glasser et al., 1991) also supports the involvement of transcriptional regulation of the SP-C gene in response to glucocorticoids. However, the possibility that post-transcriptional mechanisms are also involved cannot be ruled out. The similar alteration patterns of SP-C and SP-B mRNA contents detected in explants (26-day) in the presence of dexamethasone (10^{-8}M) indicated the possibility that a similar mechanism, which was shown in chapter 5 to be stabilization of the messenger for SP-B protein, may be involved. SP-B mRNA levels in explants treated with actinomycin D in the presence of dexamethasone were maintained at levels comparable to that in untreated culture (chapter 5, Fig.5.8). In addition, with 26 day cultures cycloheximide inhibited the dexamethasone-induced increase in SP-B mRNA content but did not affect SP-B gene transcriptional activity. These findings indicated that although the SP-C mRNA response to dexamethasone treatment was closer to SP-B mRNA than to SP-A mRNA, different mechanisms from that for SP-B mRNA may be involved in regulating SP-C mRNA levels by glucocorticoids.

The stimulatory effects of dexamethasone (10^{-8}M) on SP-C mRNA levels found with the 26-day fetal lung explants in the present study could reflect the responsiveness of type II cells to glucocorticoids, since an increase in SP-C proprotein and mRNA levels have been observed when a human adenocarcinoma cell line (H-820) was cultured with various concentrations of dexamethasone (10^{-10}-10^{-7}M) (O'Reilly et al., 1989). In addition, in vivo dexamethasone treatment of the rat (Schellhase and Shannon, 1991; Fisher et al., 1991) also resulted in an elevation of SP-C mRNA levels.

However, in vivo administration of betamethasone to the pregnant rabbit at 26-days gestation as reported by Connelly (1991) in this laboratory resulted in a decrease in SP-C mRNA levels. The reason for the discrepancy in SP-C gene expression in the rabbit between in vivo and in vitro glucocorticoid treatment is not clear. Both betamethasone and dexamethasone have the same relative potencies and long biological half-life (Haynes. Jr, 1990). It could be due to differences in duration of treatment since elevation of SP-C mRNA content was only observed after 48 h treatment of rabbit lung explants with the hormone (Fig. 6.5).

In summary, we examined SP-A and SP-C mRNA levels in developing rabbit lung and their response to dexamethasone treatment in lung explants. Results obtained with respect to SP-A and SP-C mRNA synthesis and accumulation were compared with that observed for SP-B mRNA. Potential mechanisms involved in controlling the levels of the mRNAs for the three surfactant proteins in vivo, as well as in lung explants, upon dexamethasone treatment have been discussed. It has been suggested that in normally developing fetal lung, gene expression of SP-A, SP-C and SP-B are regulated pre-

translationally. They appear to be controlled by separate mechanisms which somehow result in an coordinated increase in surfactant protein mRNA levels as the fetus approaches term. With regards to the effect of glucocorticoids, regulation of SP-A gene expression did not seem to be responsive to in vitro dexamethasone treatment, with the hormone at 10^{-8}M and lung tissue at the later stages of development. The SP-C mRNA levels responded to dexamethasone in 26-day but not 30-day lung explants in a similar but more modest manner than SP-B mRNA. The different response patterns observed at different gestational ages for the individual surfactant proteins to glucocorticoid treatment need to be confirmed and further explored.

CHAPTER 7 - SUMMARY AND FUTURE DIRECTIONS

Pulmonary surfactant is a complex mixture of phospholipids and proteins which is important in the initiation and maintenance of normal breathing by reducing surface tension at the air-aqueous interface in the lung. It is known that surfactant synthesis is under both developmental and multihormonal regulation. Surfactant replacement therapy and antenatal glucocorticoid administration have been used for the prevention of RDS, a respiratory disorder developed mostly in premature infants. Because the surfactant-associated proteins, SP-A, SP-B, SP-C and SP-D, play important roles in surfactant function, it is important to understand the mechanisms by which the expression of the surfactant proteins are regulated during fetal development and under various hormonal treatments.

The factors involved in the regulation of the most abundant surfactant protein, SP-A, are well documented. However, very little is known about the control of the other surfactant proteins. Thus, we attempted, in the studies described in this thesis, to examine and characterize the regulation of expression of three surfactant proteins, SP-A, SP-B and SP-C, in developing rabbit lung. A rabbit cDNA encoding the SP-B precursor was obtained and sequenced, as described in chapter 3. The rabbit model, which has a relatively long gestational period (31 days), was used to study the in vivo developmental patterns of the surfactant proteins. Glucocorticoid regulation of expression of these proteins was investigated in an explant system with lungs from different gestational age fetuses (26-day and 30-day). Both mRNA accumulation and synthesis for each protein

were examined under various conditions using blot analyses and nuclear transcription elongation (run-on) assays so that the regulation could be better characterized at the pre-translational level.

Comparison of the in vivo ontogenies of SP-A, SP-B and SP-C at the gene transcriptional and mRNA levels (Chapter 4) revealed a striking similarity among the three surfactant proteins during the later stages (from 28 days on) of fetal lung development. This similarity, presented as parallel alterations in mRNA transcription and mRNA steady-state levels, suggested that the three surfactant protein mRNAs may be regulated in a coordinated manner and mainly at the transcriptional level as the fetus approaches term. The fetal lung may therefore represent an excellent model for studying the regulation of several genes encoding proteins which interact to produce a physiological product.

Also implied by the comparison of the ontogenic patterns was the possibility that the levels of mRNAs for SP-A, SP-B and SP-C were regulated independently in rabbit lung before 26-days gestation. This was evident from the observation of the relatively high levels of gene transcription with SP-B whereas SP-C mRNA accumulated at a relatively high level in lungs between 22-26 days gestation. The origin of the relatively high levels of SP-B gene transcription during this period could be further investigated by isolating pretype-II cells followed by detection of SP-B mRNA levels and determination of gene transcription rates with these cells. This would allow for a comparison between alveolar and broncheolar cells in SP-B mRNA production. It should be noted that type II-cell precursors could be difficult to identify at this stage. In addition, the functional

relevance of the relatively high levels of SP-C mRNA detected in lungs of days 22 to 26 gestational rabbit fetuses will await the development of suitable antibodies directed against mature SP-C peptide which can be used for immunohistochemical studies.

Direct evidence as to whether the expression of SP-A, SP-B or SP-C is controlled by transcriptional mechanisms could be obtained using approaches such as detection of DNase I hypersensitive sites conducted with nuclei isolated from different gestational age lungs. As discussed in chapter 1, a DNase I hypersensitive site has been identified for lung-specific expression of SP-A in the rabbit (Chen et al., 1992). This site, however, appears not to relate to the developmental regulation of the gene since no alteration in the DNase I digestion pattern was apparent with nuclei isolated from rabbit lung of 21 and 28 days gestation. The in vivo information obtained by this particular approach could be further characterized by studies such as foot-printing or gel mobility shift assays using defined upstream DNA fragments obtained from the surfactant protein genes. The ability of these sequences to interact with nuclear extracts from lungs of various gestational ages would indicate the potential of these sites to function in the developmental regulation of a certain surfactant gene. Transgenic mice carrying the upstream sequences of interest from each of the genes could be used to investigate the ability of these fragments to influence gene transcription at various stages of development in vivo.

Measurement of the relative turnover rates of SP-B mRNA in lung tissue from different gestational age fetuses has been attempted. This could also be conducted for SP-A and SP-C mRNA. It should be noted that this approach may not necessarily reflect

the true situation in lung per se since, as discussed in section 5.3, circulating hormones may be involved in post-transcriptional regulation of the surfactant protein mRNAs.

The present studies on dexamethasone regulation using the lung explant system indicated that although all three surfactant proteins were responsive to glucocorticoids, differences were observed in various patterns due to glucocorticoid action (Chapters 5 & 6). First of all, the responsiveness of each gene to glucocorticoids appears to be dose-related, as indicated by the dose-dependent biphasic effects on SP-A mRNA and the elevation in the mRNAs for SP-B and SP-C but not for SP-A in lung explants cultured with 10^{-8}M dexamethasone in the time course studies. Secondly, the effect of glucocorticoids on these three genes appear to be age-related, with more pronounced effects observed with 26-day lung explants compared to 30-day explants. There also seems to be an optimal window for the surfactant proteins response to the hormone treatment. Preliminary experiments using 21-day rabbit fetal lungs demonstrated that smaller elevations in the levels of the mRNAs for SP-B and SP-C were observed in the presence of 10^{-8}M dexamethasone as compared to those with 26-day explants, although similar patterns were found with respect to the direction of the hormone effects. Again, no significant response was observed in SP-A mRNA levels or gene transcription rates in 21-day lung explants after application of this particular concentration of dexamethasone. Third, the manner in which glucocorticoids regulate mRNA levels for each surfactant protein appears to be different. As demonstrated in Chapter 5, dexamethasone increased SP-B mRNA in vitro mainly through post-transcriptional mechanisms. This could also be true for regulation of SP-C mRNA abundance in lung

explants (Chapter 6). In addition, transcriptional mechanisms might also be involved in the regulation of SP-C mRNA levels. This was indicated by the dose-response studies where increases, although small, were apparent in SP-C gene transcription with day 26 lung explants with increasing concentrations of dexamethasone applied.

It would be useful to examine the functional relevance of the elevations in SP-B and SP-C mRNA levels, observed with 26 day lung explants, in response to in vitro dexamethasone (10^{-8}M) treatment. We attempted to measure the relative SP-B protein contents in the fetal rabbit lung explants using a specific monoclonal antibody directed against bovine mature SP-B (chapter 5). Although a positive band was detected from homogenates of the explants, the protein does not appear to be SP-B. However, this needs to be confirmed with another heterologously generated antibody against SP-B. The significance of the increased SP-C mRNA levels observed with glucocorticoid treatment must await the availability of a specific antibody or another suitable assay.

Based on these results and those reported previously, we proposed that it could be important (1) to define the window with respect to gestational age when the expression of SP-A, SP-B and SP-C start to become regulated coordinately in animal models such as the rabbit and rat; (2) to investigate the optimal concentration of certain glucocorticoids, such as dexamethasone or betamethasone, where positive effects of the hormone on all three surfactant proteins would be observed. For studies using rabbit lung explants, 10^{-9}M of dexamethasone could be appropriate, according to results shown in Fig. 6.3 and 6.4. (3) to investigate the effects of some paracrine and autocrine factors, such as cyclic AMP and its analogues, EGF, TGF-β, TNF-α with respect to a

defined window which is correlated with gestational age and duration of glucocorticoid treatment. These would help define the role of the cellular environment in modulating the program of hormone responses under various conditions. Together with elucidation of the relationship between glucocorticoids and other circulating hormones such as thyroid hormone and insulin, these would provide very useful knowledge for the design of a most efficient preventive regimen for NRDS. At the same time, this would further our understanding of biological development and its regulation in mammalian system.

APPENDIX I

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THE RESTRICTION MAP FOR DNA SEQUENCE OF RABBIT SP-A cDNA

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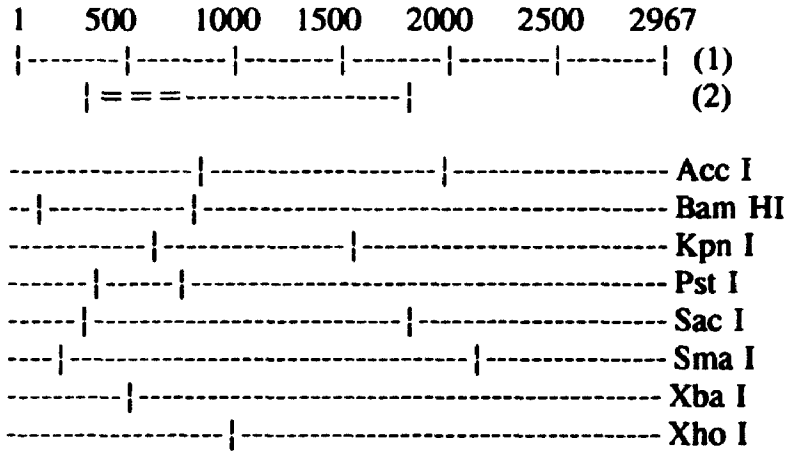


Sites of Cuts (Clone 1):

Acc I:	873 2033
Bam HI:	163 829
Kpn I:	690 1691
Sac I:	374 1877
Sma I:	242 2241
Xba I:	555
Xho I:	1030

List of non-cutting selected enzymes.

Eco RI , Eco RV , Hind III , Hpa I , Pvu II , Sac II ,Sph I

Clone Information.

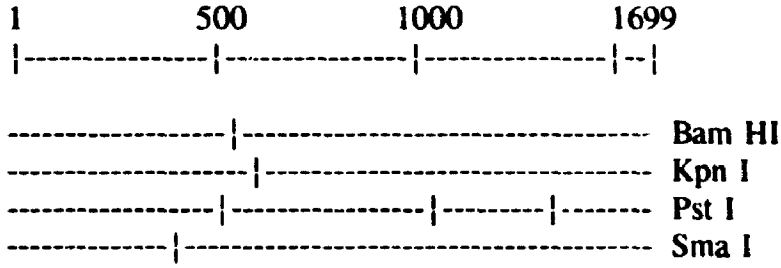
Clone (1): Boggaram & Mendelson, JBC, 263:2939-2947, 1988
Clone (2): Connelly, Ian (Dr. F. Possmayer's Lab)

vector:	Bluescript.SK$^+$, pUC19 (clone 2)
cloning site:	Eco RI
insert size:	1400 bp (with coding sequence of 400 bp "=" at the 5' end)

****

THE RESTRICTION MAP FOR DNA SEQUENCE OF RABBIT SP-B cDNA

****



Sites of Cuts:

Bam HI:	610
Kpn I:	675
Pst I:	594 1219 1427
Sma I:	446

List of non-cutting selected enzymes.

Eco RV ,Hind III ,Hpa I ,Not I ,Sac II ,Xba I ,Xho I

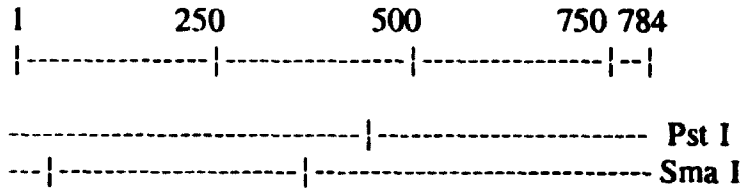
Clone Information.

vectors:	Bluescript.SK$^+$, pUC19
cloning site:	EcoRI
insert size:	1700 bp

****

THE RESTRICTION MAP FOR DNA SEQUENCE OF RABBIT SP-C cDNA

****



Sites of Cuts:

Pst I: 444
Sma I: 67 356

List of non-cutting selected enzymes.

Acc I , Bam HI , Eco RI , Eco RV , Hind III , Hpa I , Kpn I ,Not I , Pvu II , Sac I , Sac II , Sph I , Xba I , Xho I

Clone Information.

vectors: Bluescript.SK$^+$, pUC19
cloning site: Eco RI
insert size: 800 bp

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